

STUDY OF THE ACTIVATION OF PERIPHERAL BLOOD AND CORD BLOOD NATURAL KILLER CELLS

Rehab Mousa Alnabhan

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Anthony Nolan Research Institute
Cancer Institute
University College London (UCL)

Declaration

I, Rehab Mousa Alnabhan, confirm that the work presented in this thesis is my own.
Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:.....

Date:.....

*“This thesis is dedicated to my parents for
their endless love and support”*

Abstract

Background

Natural killer (NK) cells are cytotoxic effectors providing a first line of defence against viruses and tumours. NK cells can be isolated from peripheral blood (PB) or cord blood (CB) for cancer immunotherapy. However, it was shown by our group and others, that CB NK cells express higher NKG2A and less killer immunoglobulin like receptors (KIRs) than PB NK cells indicating an immature phenotype. Also, CB NK cells require high doses of interleukin (IL)-2 for proliferation and activation. It was also shown that resting CB NK cells are poorly cytotoxic and produce less IFN- γ than PB NK cells after stimulation with IL-2.

Hypothesis and aims

CB NK cells have an immature phenotype and could mediate different role in neonatal immunity than adult PB NK cells. Hence, the aim of this study was to explore whether differential mechanisms of activation exist between PB and CB NK cells.

Methods

PB samples from healthy volunteers and CB samples were obtained with prior written consent and ethical approval from Anthony Nolan. Purified PB and CB NK cells were stimulated with cytokines including IL-2, IL-12, IL-15, IL-18, individually or in combination. Thereafter, comparative analysis was performed on their phenotype, signalling, proliferation, cytotoxicity, cytokine secretion and chemotaxis post-cytokine stimulation.

Results

My results show that CB NK cells responded less to IL-2 activation than PB NK cells, which correlated with lower levels of IL-2 receptors and decreased phosphorylation of STAT5 pathway. CB NK cells activated with IL-15+IL-2 showed enhanced cytotoxicity while activation with IL-15+IL-18 promoted maximal proliferation, higher IFN- γ and TNF- α secretion. In contrast, optimal activation of PB NK cells was achieved by IL-2 stimulation. Interestingly, CB NK cells secreted substantial IL-8

concentrations following cytokine stimulation. IL-12 or IL-18 stimulation induced L-selectin expression on CB NK cells and promoted NK cell migration towards chemokines that induce homing to lymph nodes. I also generated long-lived memory-like NK cells from PB and CB using cytokines whereby IL-12+IL-15+IL-18 pre-activation led to substantial IFN- γ production.

Conclusions

CB NK cells are fully functional upon activation with IL-15+IL-2 or IL-15+IL-18 rather than IL-2 thereby providing a basis for activation of NK cells derived from different sources that may be utilised for future NK cell-based therapeutic purposes. In addition, CB NK cell cytokine secretion profile is suggestive for a role of neonatal NK cells in providing protective immunity against bacterial infections.

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Abbreviations

⁵¹ Cr	⁵¹ Chromium
7AAD	7-Amino-actinomycin D
aAPC	Artificial antigen presenting cells
ACTB	Actin-beta
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICD	Activation-induced cell-death
AICL	Activation-induced C-type lectin
AML	Acute myeloid leukemia
APCs	Antigen presenting cells
BAT3	HLA-B-associated-transcript 3 (BAT3)
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
Ca ²⁺	Calcium
CB	Cord blood
CBMCs	Cord blood mononuclear cells
CCL	C-chemokine ligand
CCR	C-chemokine receptor
CD	Cluster of differentiation
CD62L	L-selectin
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule
CFSE	Carboxyfluorescein succinimidyl ester
Chem23	Chemrin23
CHS	Contact hypersensitivity
CLL	Chronic lymphocytic leukemia
CLPs	Common lymphoid progenitors
CMV	Cytomegalovirus
CTL	Cytotoxic T cell
CXCL	CX-chemokine ligand
CXCR	CX-chemokine ligand
DAP12	12 kD signal-transducing adapter
DCs	Dendritic cells
DD	Death domain
dNK cells	Decidual NK cells
DR	Death receptor
E:T	Effector: Target
EBV	Epstein Bar virus
ER	Endoplasmic reticulum
FBS	Foetal bovine serum

FCS	Foetal calf serum
FLT3	Fms-related tyrosine kinase 3
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GMP	Good manufacturing practice
GvHD	Graft versus host disease
GvL	Graft versus leukemia
h	Hour
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEV	High endothelial venules
HIS	Human immune system
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILTs	Ig-like transcripts
iNKT	Invariant NKT cells
IONO	Ionomycin
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
IU	International unit
JAK	Janus tyrosine-kinase
KIR	Killer-cell immunoglobulin-like receptors
KLRG1	Killer cell lectin-like receptor G1
LAK	Lymphokine activated killer
LFA-1	Lymphocyte function-associated antigen
LIRs	Leukocyte Ig-like receptors
LLT1	Lectin-like transcript-1
LN	Lymph node
LT α	Lymphotoxin α
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MICA	MHC class I-related chain A molecule
MICB	MHC class I-related chain B molecule
Min	Minute
mL	Milliliter

MML5	Mixed-lineage leukemia-5 protein
mPB	Mobilised peripheral blood
mRNA	Messenger RNA
NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear factor-κB
ng	Nanogram
NK cells	Natural killer cells
NKPs	Natural killer progenitors
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pc	Pico gram
PCR	Polymerase chain reaction
Pen-Strep	Penicillin Streptomycin mixture
PMA	Phorbol 12-myristate 13-acetate
PPRs	Antigen-specific pattern recognition receptors
PVR	Poliovirus
SCF	Stem cell factor
Sec	Second
Scid	Severe combined immunodeficiency
SLAM	Signaling lymphocyte activation molecule
STAT	Signal transducer and activator transcript
TBI	Total body irradiation
TCR	T cell receptor
T _H	T helper
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TRAF2	TNF-receptor-associated factor 2
TRAIL	TNF-related apoptosis induced ligand
Tregs	Regulatory T cells
UBC	Ubiquitin
V-D-J	Variable-Diversity-Joining
VEGF	Vascular endothelial growth factor
β-ME	β-Mercaptoethanol
β2M	Beta-2 microglobulin
γc	Gamma chain
μL	Microliter

Chapter 1: Introduction

1.1 The immune system

The immune system is a complex system composed of immune cells, tissues and molecules that work cooperatively to eradicate infection and compromised host cells. The immune system is divided into two main categories, innate immunity and adaptive immunity.

1.1.1 The innate immune system

The innate immune system is the first line of defence against pathogens. The components of the innate immune system include epithelial barriers, phagocytes, dendritic cells (DCs), natural killer (NK) cells, the complement system and cytokines. Importantly, there is no specific memory in the innate immune system. Innate immune cells respond to microbes in a non-specific manner and are able to discriminate between self and non-self using pattern recognition receptors (PRR). Innate immune cells recognise molecular patterns shared by specific groups of microbes that are not found on host cells via germ-line encoded PRRs such as Toll-like receptors (TLRs). These receptors have limited diversity, as they do not undergo somatic rearrangement (Medzhitov and Janeway, 2002). In response to pathogens, innate immune cells release inflammatory cytokines such as interleukin (IL)-12 and tumour necrosis factor (TNF) that provide activation signals for cells of the innate and adaptive immune systems (Table 1.1) (Abbas and Lichtman, 2009). As NK cells are the focus of this study, they will be discussed in more details in subsequent sections.

Table 1.1 List of cytokines and their effects

Cytokine (s)	Source (s)	Effect (s)	Reference(s)
IFN- α	DCs and macrophages	NK cell activation Promotes anti-viral effects and increases MHC class I expression	(Costa et al., 2009)
IFN- β	Fibroblasts	NK cell activation Promotes anti-viral effects and increases MHC class I expression	(Iversen et al., 2005)
IFN- γ	NK cells	Macrophage activation Anti-viral protection Tumour growth inhibition	(Schroder et al., 2004)
IL-1	Macrophages, endothelial cells Some epithelial cells	Production by endothelial cells promotes inflammation and coagulation Synthesis of acute phase proteins by the liver	(Goldbach-Mansky, 2012)
IL-6	Macrophages, endothelial cells T cells	Synthesis of acute phase proteins by the liver Proliferation of antibody-producing cells	(Eto et al., 2011, Hall et al., 2010)
IL-8	Macrophages, endothelial cells Some epithelial cells	Chemotaxis, angiogenesis Neutrophil migration towards the inflammatory sites	(Roda et al., 2006, El-Shazly and Lefebvre, 2011)
IL-10	Macrophages and DCs Treg and T _H 2	Inhibition of IL-12 production Decreases the expression of MHC class I	(Saraiva and O'Garra, 2010)
IL-12	Macrophages and DCs	Promotes the production of IFN- γ by NK cells and T cells and increases their cytotoxicity Mediates T cell differentiation into T _H 1 cells	(Marcenaro et al., 2005) (Smyth et al., 2000)
IL-15	Thymus and BM stromal cells Macrophages and DCs	NK cell development and proliferation T cell proliferation	(Liu et al., 2000, Ranson et al., 2003, Waldmann, 2006)
IL-18	Macrophages	Induces IFN- γ production by NK cells and T cells NK cell migration	(Agaugue et al., 2008, Mailliard et al., 2005)
TNF	Macrophages and T cells NK cells	Endothelial cell inflammation and coagulation, neutrophil activation Synthesis of acute phase proteins, apoptosis	(Sethi et al., 2009)

BM (bone marrow), IFN (interferon), IL (interleukin), T helper 1 (T_H1), TNF (tumour necrosis factor), MHC (major histocompatibility complex).

1.1.2 The adaptive immune system

If the innate immune response fails to eliminate the pathogen or transformed cells, a highly specific immune response needs to be mounted by the adaptive immune system. An adaptive immune response is elicited when an antigen crosses the epithelial barrier and is presented within lymphoid organs. This response is antigen specific, systemic and is characterised by immunological memory, which is the ability of the immune system to respond more rapidly and effectively to pathogens if re-encountered. The adaptive immune system comprises T and B lymphocytes as well as their products. Furthermore, adaptive immunity is further subdivided into humoral or cell-mediated immunity. Antibodies produced by activated B cells, which are also called plasma cells, mediate humoral immunity. Antibodies function by blocking and neutralising extracellular microbes and toxins whereas intracellular microbes such as bacteria and viruses can proliferate inside host cells and need to be destroyed by cell-mediated immunity. This type of response involves antigen specific cytotoxic T cells that leads to the destruction of infected cells (Abbas and Lichtman, 2009, Parkin and Cohen, 2001)

1.1.2.1 Humoral immunity

Antibodies released by B cells mediate the humoral immune response. There are different antibody classes categorised according to their heavy chain type including immunoglobulin (Ig) A, IgD, IgE, IgG and IgM. Each one has different functions and distribution within the body. Antibodies released into the circulation mediate several functions including neutralisation of extracellular microbes and microbial toxins, activation of the complement system and opsonisation of target cells that will be eliminated by antibody-dependent cell-mediated cytotoxicity (ADCC). Humoral immune responses are characterised by the generation of high-affinity, class-switched antibodies against specific antigens and long-lived antibody-secreting cells with specific memory and the ability to respond rapidly to subsequent antigen exposure (Janeway et al., 2001).

Circulating naïve B cells express membrane-bound IgG and IgM that help mediate immune surveillance. A peptide-specific humoral response is elicited when B cells that

reside in the follicles of lymphoid organs interact with T cells. In this context, B cells act as antigen presenting cells (APC) via MHC class II molecules presenting antigens to T cells. This results in B cell activation and proliferation of antigen-specific B cells that further differentiate into specific plasma cells that secrete antibodies. The specific cytokines present in the milieu dictate the nature of heavy chain class switching. The presence of IFN- γ leads to the generation of IgG (mediating complement activation and phagocytosis), whereas the presence of IL-4 leads to the generation of IgE (mediating activation of hypersensitivity or helminthic immunity) (Mosmann and Sad, 1996).

1.1.2.2 Cell-mediated immunity

T cells are the primary mediators of cell-mediated immunity. T cells are divided into two main types: T helper cells (T_H or CD4) and cytotoxic T cells (CTL or CD8). T cells are important for the clearance of pathogens that are ingested by phagocytic cells or that are living within infected cells. T cell responses require the recognition of cell-associated antigens, which results in the proliferation of antigen-specific clones and differentiation of effector and memory cells (Abbas and Lichtman, 2009).

T cell activation is MHC-restricted where T cells are only able to recognise and respond to specific antigens displayed by MHC molecules. MHC molecules are polymorphic and are composed of two main classes; MHC class I which is expressed by all nucleated cells and MHC class II which is expressed by macrophages, DCs and B cells. Both classes contain a peptide binding cleft on their outer surface allowing presentation of antigens to T cells. T cells express a T cell receptor (TCR) that recognises peptides bound to MHC molecules on APCs. B cells, macrophages and DCs are known as professional APCs as they capture antigens with high efficiency for presentation to T cells. CD4 T cells recognise peptides presented via MHC class II molecules whereas CD8 T cells recognise peptides presented by MHC class I molecules (Parkin and Cohen, 2001). Upon activation, naïve CD4 T cells differentiate into specialised T_H1 cells (important for the response to intracellular microbes), T_H2 cells (important for the response to extracellular microbes) (Mosmann and Sad, 1996), T_H17 cells (that produce IL-17 which is involved in inflammation and autoimmune diseases) (Ouyang et al., 2008), or regulatory T cells (key in immunosuppression and self-tolerance) (Yamaguchi and Sakaguchi, 2006). The presentation of peptides by MHC class I molecules to CD8

T cells results in differentiation of CTLs and elimination of infected cells. Both processes are regulated by a requirement for a second signal to be delivered via the T cell receptor (TCR) and co-stimulatory molecules, in particular B7 binding to CD28 on naïve T cells (Abbas and Lichtman, 2009). In addition, a third signal delivered by cytokines produced by innate immune cells is required to drive T cell polarisation such as IFN- γ being produced by NK cells (Corthay, 2006).

Within two days post-antigen exposure, antigen-specific effector cells proliferate and undergo clonal expansion. After clearance, a fraction of T cells survive and differentiate into long-lived memory T cells. These cells are quiescent in steady state but rapidly respond to subsequent exposure to the same antigen. There are two types of memory T cells: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} produce high levels of IL-2 and are characterised by the expression of CCR7, L-selectin and CD45RO, hence this phenotype allows them to reside in lymphoid organs and mediate clonal expansion upon secondary exposure. Conversely, T_{EM} do not express CCR7 but express other chemokine receptors such as CCR4, CCR5 and CXCR3 that are involved in homing to inflamed tissues. As such, T_{EM} are present in mucosal tissues and are responsible for rapid effector function upon restimulation and production of inflammatory cytokines such as IFN- γ or IL-4 (Sallusto et al., 2004).

1.2 Natural killer cells

Natural killer (NK) cells were first described in mice in 1975 as granular lymphoid cells with cytotoxic function against Moloney leukaemia cells (Kiessling et al., 1975). The same year, Herberman *et al.* confirmed the existence of effector cells that were cytolytic against tumour cells and were not T cells using athymic nude mice (Herberman et al., 1975). Subsequently, these cells were identified as NK cells due to their ability to induce natural cytotoxicity without prior sensitisation. Unlike T and B cells, NK cells do not undergo germ-line Variable-Diverse-Joining (V-D-J) receptor rearrangement, and hence, are classified as a component of the innate immune system (Lanier et al., 1986b). NK cells are currently defined as large granular lymphocytes, comprising about 5 - 15 % of total mononuclear cells in peripheral blood (PB).

1.2.1 NK cell subsets

NK cells are characterised by the expression of CD56 and absence of CD3 (Ritz et al., 1988). Circulating NK cells are divided into two major subsets based on the intensity of CD56 expression: CD56^{bright} and CD56^{dim} NK cells (Fig 1.1); each of which has distinct phenotypic characteristics and functions (Lanier et al., 1986a). In addition, an aberrant NK cell population, which is CD56^{neg}, can be found in small percentages in healthy individuals, but expand as a result of chronic viral infections such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (Björkström et al., 2010).

1.2.1.1 CD56^{bright} NK cells

CD56^{bright} NK cells comprise about 10 % of total circulating NK cells. CD56^{bright} NK cells are cytokine producers, releasing various cytokines including IFN- γ , TNF- α , TNF- β and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig 1.1 A). These pro-inflammatory cytokines play key roles in recruiting and priming other immune cells. CD56^{bright} NK cells do not express CD16 or express low levels of this receptor (Cooper et al., 2001c). CD56^{bright} NK cells express negligible amounts of killer immunoglobulin like receptors (KIR) (Andre et al., 2000). However, CD56^{bright} NK cells exhibit high expression of the lectin-like inhibitory receptor CD94/NKG2A (Voss et al., 1998) and also express high levels of L-selectin, CCR7 and CXCR3, which are chemokine receptors associated with homing to lymph nodes (LN) (Campbell et al., 2001). Moreover, CD56^{bright} NK cells express the high affinity IL-2 receptor complex, IL-2R $\alpha\beta\gamma$ (Caligiuri et al., 1990). As a result, this subset is responsive to very low doses of IL-2, thereby displaying higher proliferative capacity *in vitro* and *in vivo* compared to CD56^{dim} NK cells.

1.2.1.2 CD56^{dim} NK cells

CD56^{dim} NK cells constitute up to 90 % of total NK cells in the periphery. These cells are characterised by the abundant expression of the CD16 (Fc γ III) receptor, a receptor that mediates antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig 1.1 B). Additionally, CD56^{dim} NK cells kill target cells by releasing perforin and granzyme by granule exocytosis or by inducing apoptosis following activation of the TNF-related apoptosis induced ligand (TRAIL) or Fas/FasL cell death pathways. Phenotypically,

CD56^{dim} NK cells are also characterised by the high expression of KIR. CD56^{dim} NK cells have significantly lower or scarce expression of L-selectin (CD62L) when compared to CD56^{bright} NK cells (Frey et al., 1998). Unlike CD56^{bright} NK cells, resting CD56^{dim} NK cells express the heterodimeric intermediate affinity IL-2 receptor (IL-2R $\beta\gamma$) and require higher doses of IL-2 for proliferation. Moreover, CD56^{dim} NK cells express high levels of LFA-1, which is key for the formation of conjugates with target cells. In addition, CD56^{dim} NK cells express CXCR1 and CX3CR1, which are chemokine receptors involved in NK cell trafficking towards non-lymphoid inflamed tissues (Cooper et al., 2001a).

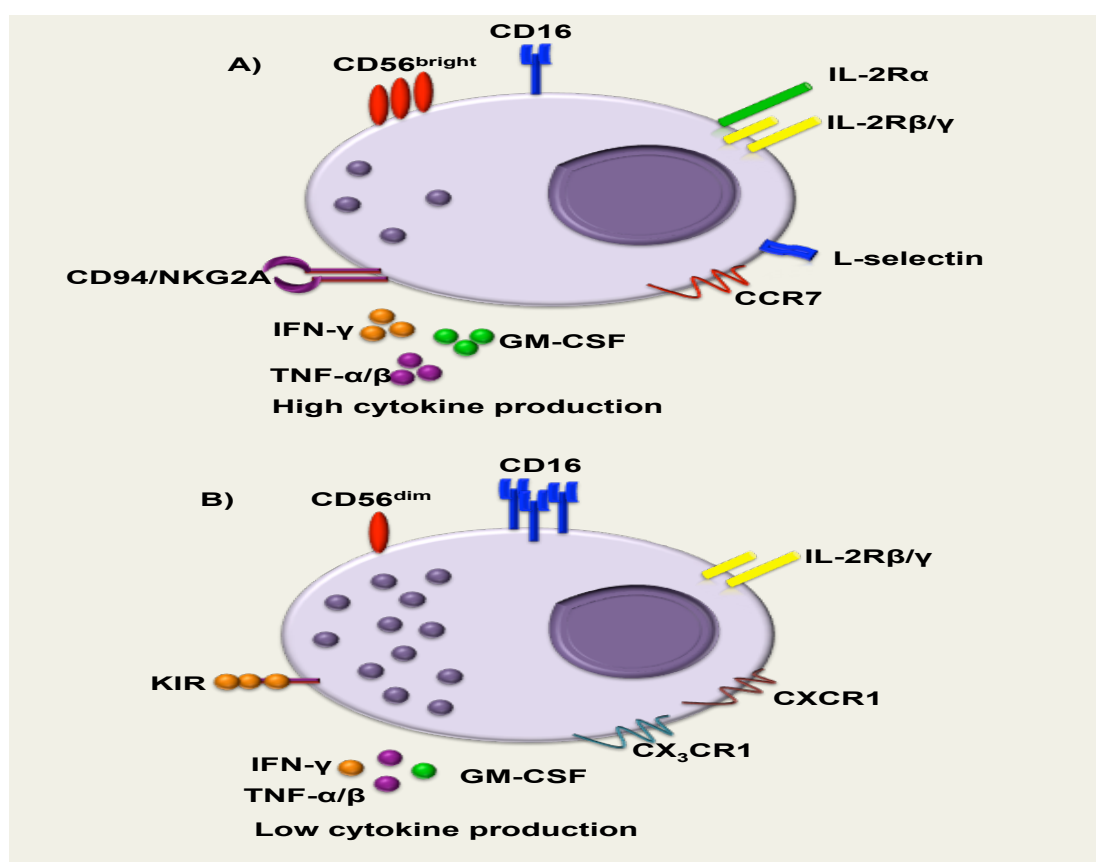


Figure 1.1 Human peripheral blood circulating NK cell subsets. A) CD56^{bright} NK cells are potent producers of inflammatory cytokines. B) CD56^{dim} NK cells are characterised by their high expression of CD16 and of granzymes and perforin, both of which are involved in the cytotoxic function of NK cells (Cooper et al., 2001a). License obtained from Elsevier; 3505490975127

1.2.2 NK cell development

NK cell development and differentiation occurs in the bone marrow (BM), however, other sites of NK cell differentiation have been described. Vosschenrich *et al.* reported

the existence of thymus-derived NK cells characterised by CD127 and GATA-3 expression (Vosshenrich et al., 2006), which have been recently classified as type 2 innate lymphoid cells (ILC2) (Vosshenrich and Di Santo, 2013). A distinct CD56^{bright} population derived from CD34^{dim}CD45⁺ cells has been found in lymph nodes (LNs) (Freud et al., 2005). Furthermore, CD34⁺ cells present in the human decidua can give rise to functionally distinct NK cells that are involved in immunomodulation and tissue remodelling (Vacca et al., 2011). Nonetheless, the consensus is that NK cells develop in the BM from CD34⁺ haematopoietic stem cells (HSC) and that NK cells present in other organs represent a very low percentage that are indicative of less mature NK cells or of distinct lineages of NK cells (Freud and Caligiuri, 2006).

Common lymphoid progenitors (CLP) generated from CD34⁺ HSC can differentiate into any lymphoid lineage. Commitment to the NK cell lineage is dependent on the acquisition of the IL-2/IL15R β (CD122) and of IL-2/IL-15R γ (CD132). The expression of these common γ chain receptors renders NK cell progenitors (NKPs) responsive to IL-15, which is abundantly present in the BM microenvironment (Colucci et al., 2003). Although exogenous IL-15 drives NK cell differentiation *in vitro*, IL-15 is mainly trans-presented *in vivo* by BM-derived accessory cells as a membrane-bound protein coupled to the IL-15R α . Using a humanised mouse model, Huntington and colleagues showed that the administration of IL-15R agonist (human IL-15 pre-incubated with IL-15R α) induces NK cell maturation and homeostasis *in vivo* as compared to administration of IL-15 alone (Huntington et al., 2009). Therefore, there are two main characteristics that define NKPs; the lack of expression of surface antigens associated with other lymphoid lineages such as CD3 or CD19 and the expression of IL-2/IL15R β that marks the transition from CLP into the NK cell lineage. Stage 1 of NK cell differentiation is characterised by expression of CD34, CD45RA and CD10. As NKPs become responsive to IL-15 they differentiate into pre-NK cells (stage 2) that express CD117 and downregulate CD10 expression (Fig 1.2). However, the surface expression of IL-2/IL15R β remains undetectable by flow cytometry during early stages of differentiation (stage 1-3) (Freud et al., 2006).

Pre-NK cells give rise to stage 3 immature NK (iNK) cells that are committed to the NK cell lineage and are no longer capable of developing into other lymphoid cells. iNK cells express CD56 and some NK cell receptors such as CD161, NKp44 and 2B4.

These cells are committed to the NK cell lineage, but lack several of the main features of NK cells. Firstly, iNK cells do not produce IFN- γ nor do they mediate killing of MHC class I negative targets in a perforin-dependent manner (Freud et al., 2006). Alternatively, iNK cells produce type II cytokines (e.g. IL-5 and IL-13) upon stimulation with high doses of IL-2 and kill target cells by activation of the TRAIL pathway (Zamai et al., 1998). In addition, iNK cells lack expression of some key NK cell receptors that are involved in the direct recognition of compromised targets including NKG2D, KIR, NKp46 and CD16 (Freud et al., 2006).

Next, iNK cells differentiate into CD56^{bright} NK cells (stage 4). At this stage, the acquisition of several NK cell receptors occurs concomitantly with the loss of CD117 and CD34 expression. CD56^{bright} NK cells express NKG2D, NKp46, exhibit intracellular expression of IFN- γ and perforin, and express high levels of CD94/NKG2A. As NK cells proceed to stage 5 of differentiation, they display the most mature NK cell phenotype, CD56^{dim}CD16^{bright}KIR⁺, and show lower CD94/NKG2A expression than stage 4 CD56^{bright} NK cells (Freud et al., 2006).

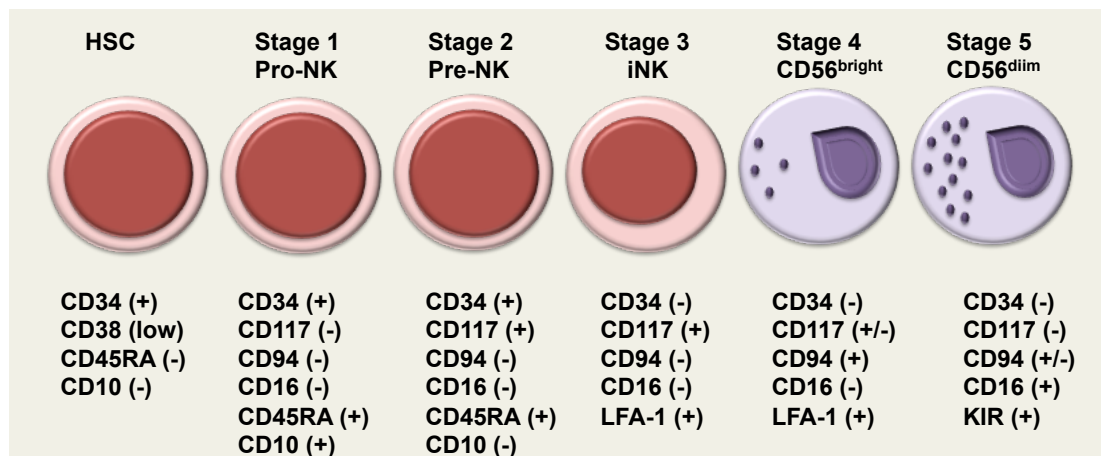


Figure 1.2 Human NK cell development stages *in vivo* as proposed by Freud and Caligiuri (Freud and Caligiuri, 2006). CD34⁺ HSCs differentiate into NK cells via distinct developmental stages. The progression from stage 1 to stage 3 dictates commitment to the NK cell lineage. Stages 4 and 5 represent where NK cells gain functional maturation that are defined as CD56^{bright} and CD56^{dim} NK cells and constitute circulating PB NK cells. License obtained from John Wiley and Sons; 3397700678902.

1.2.3 Missing-self hypothesis

The missing-self hypothesis was first proposed by Klas Karre and colleagues who noticed that the loss of self-MHC expression by murine lymphoma cells triggers NK cell effector function (Karre et al., 1986). The basis of this hypothesis is that the engagement of self-MHC molecules with inhibitory receptors such as killer Ig-like receptors (KIR) and CD94/NKG2A in humans or the lectin-like Ly49 receptors in mice, results in inhibition of NK cell cytotoxic function. Thus, NK cells attack target cells that lose self-MHC expression while those that express self-MHC will be spared. However, it has also now been proven that triggering of NK cell effector function actually requires an additional signal delivered by an activating receptor (Raulet, 2006).

1.2.4 NK cell education

During differentiation, NK cells undergo education. NK cells become fully competent in distinguishing between self and non-self by interaction with MHC class I in the BM microenvironment. This hypothesis was proposed by Kim and colleagues after the detection of impaired NK cell effector functions in MHC class I-deficient hosts (Kim et al., 2005). NK cell education involves positive engagement of NK cell inhibitory receptors with self-MHC class I molecules during maturation and is key for NK cells to become functionally responsive (licenced) (Kim et al., 2005). Thus, licenced NK cells are inhibited by self-MHC class I molecules that they encounter during maturation, which prevents them from attacking healthy cells. In contrast, unlicensed NK cells are hypo-functional.

However, the absence of expression of self-inhibitory receptors on NK cells was observed in mice (Fernandez et al., 2005) and led to a new concept known as the arming/disarming of NK cells (Fig 1.3 A, B). In the arming model, positive engagement of inhibitory receptors expressed by NK cells with MHC class I is a requirement for the induction of functionally mature NK cells. Thus, the function of NK cell inhibitory receptors during development differs from their function in mature NK cells. Accordingly, cells that fail to interact with MHC class I during maturation such as in a MHC class I deficient host are “un-armed” and hence do not attack MHC class I-negative targets (Fig 1.3 A). Conversely, according to the disarming model NK cells are

activated by default and the expression of inhibitory receptors rescue NK cells from becoming hyporesponsive (Fig 1.3 B) (Raulet and Vance, 2006). Therefore, the lack of expression of inhibitory receptors for self-MHC by developing NK cells alongside prolonged activation by an activating ligand results in hyporesponsiveness of NK cells due to continuous stimulation, a phenomenon similar to T and B cell anergy (Raulet, 2006).

Another model of education suggests that MHC class I molecules interact with inhibitory receptors within the same cell in a *cis*-interaction manner (Fig 1.3 C). It is hypothesised that some inhibitory receptors such as Ly49A deliver an inhibitory signal even in the absence of interaction with its ligand and that unengaged inhibitory receptors accumulate at the NK cell/target cell interface in the immunological synapse. Thus, *cis*-interaction sequesters inhibitory Ly49A receptors restricting interaction with target cells and preventing the relocation of inhibitory receptors to the immunological synapse. As a result, inhibition via Ly49A receptors is reduced and NK cells become more responsive (Chalifour et al., 2009).

Finally, in the rheostat model (Fig 1.3 D), NK cell responsiveness is adjusted in a quantitative manner based on the number of inhibitory receptors they express. Early encounter of NK cells with healthy cells during development dictates their threshold of responsiveness. If NK cells lack the expression of self-MHC inhibitory receptors, NK cell responsiveness will be tuned down. Conversely, NK cell responsiveness is tuned up when NK cells express one or more inhibitory receptors. Hence, NK cell responsiveness is tuned to an optimal point of balance based on the inhibitory and activating signals it receives from healthy cells. This model of NK cell education ensures self-tolerance as well as the detection of any changes occurring within healthy cells (Joncker et al., 2009).

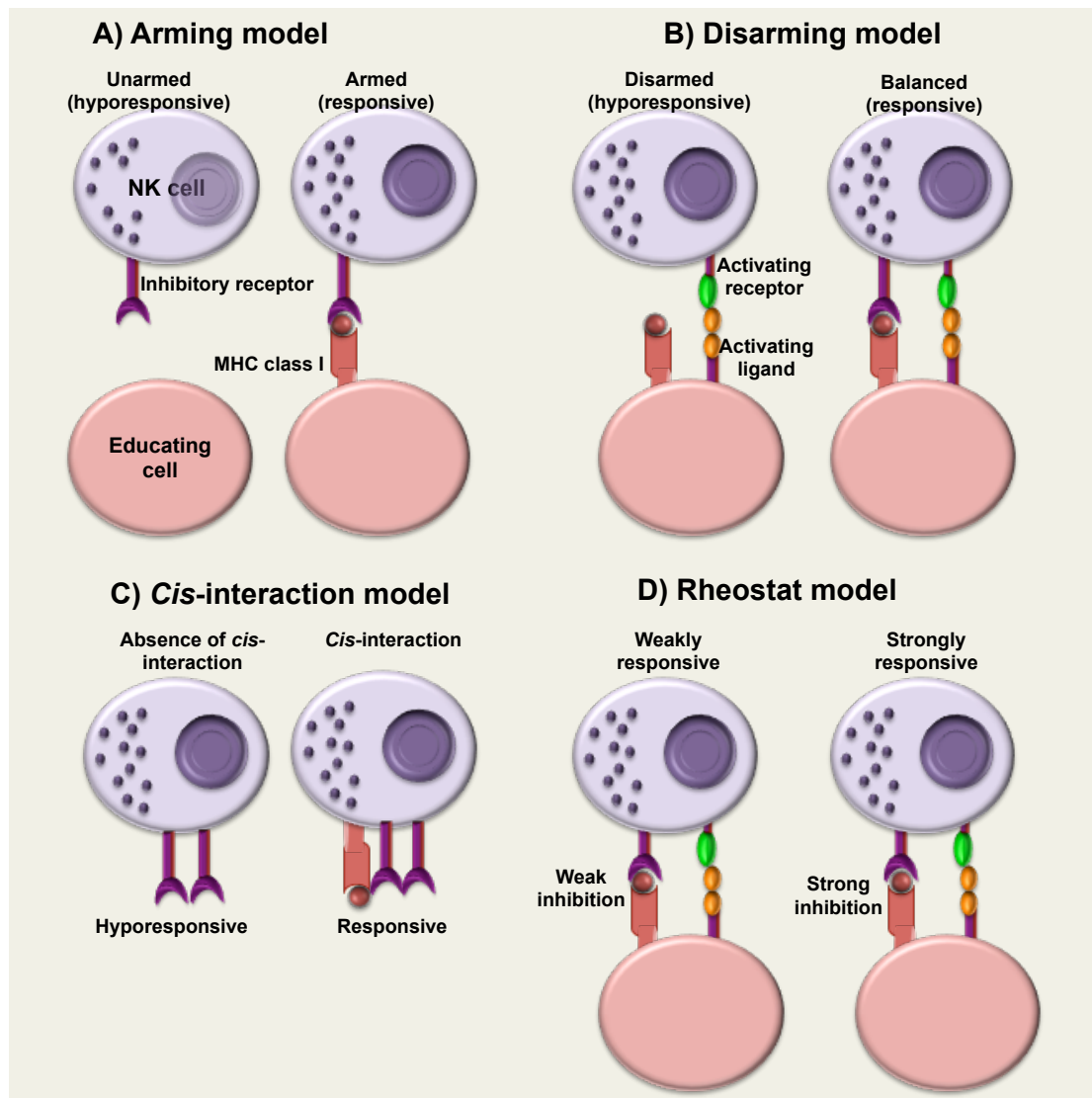


Figure 1.3 Models of NK cell education. A) The arming model states that the arming of NK cells requires positive engagement of inhibitory receptors with MHC class I on educating cells, whereas the lack of interaction results in NK cell hyporesponsiveness. B) The disarming model states that disarming of NK cells occurs as a result of the lack of expression of inhibitory receptors, thus persistent stimulation of NK cells leads to hyporesponsiveness of NK cells. C) The *cis*-interaction model states that *cis*-interaction between inhibitory receptors and MHC class I molecules on the same cell sequesters the inhibitory signal at the immunological synapse. D) The rheostat model suggests that the strength of responsiveness of NK cells depends on the strength of inhibition delivered by inhibitory receptors (Höglund and Brodin, 2010). License obtained from Nature Publishing Group; 3397700260392.

1.2.5 NK cell receptors

NK cell functions are regulated by balancing signals delivered by inhibitory and activating receptors. Accordingly, a target cell that downregulates MHC class I expression or upregulates stress ligands will trigger NK cell effector function (Fig 1.4).

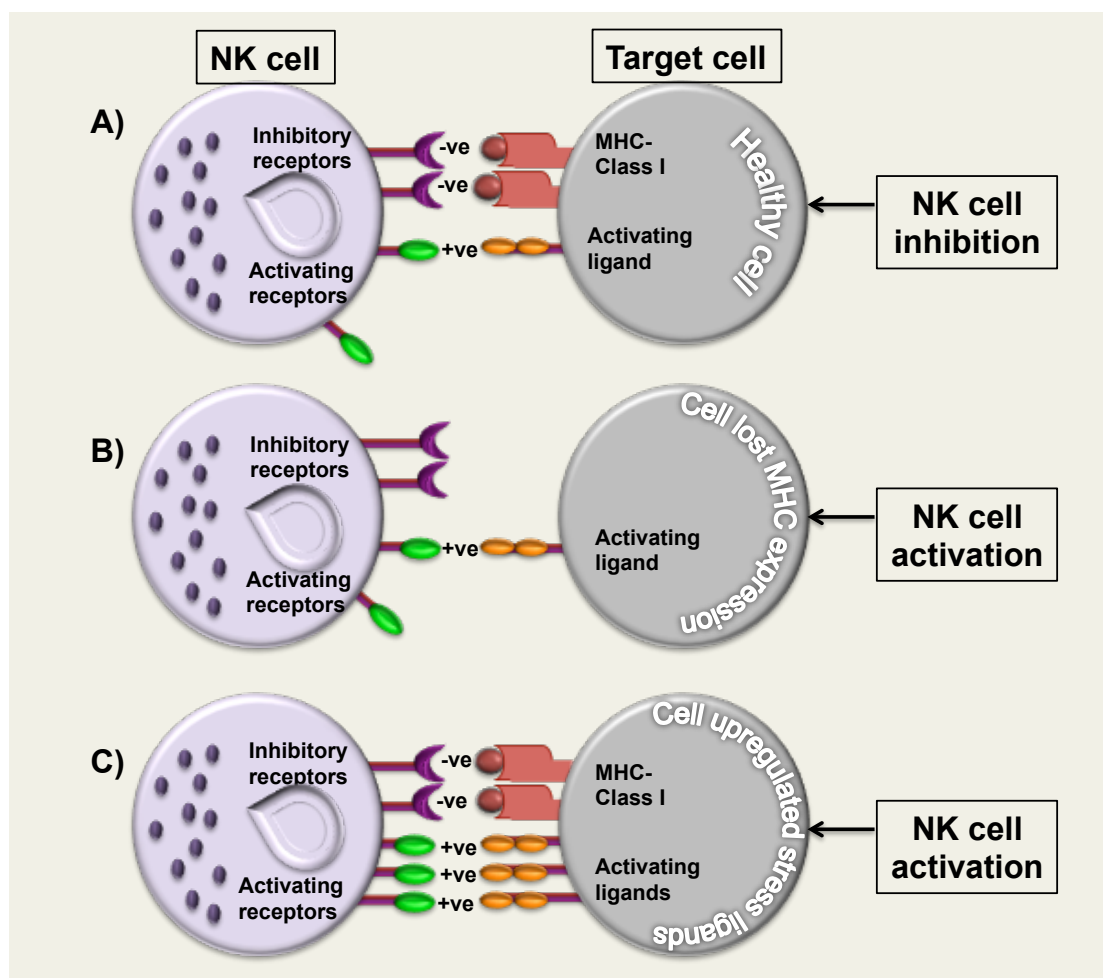


Figure 1.4 Regulation of NK cell recognition of target cells by activating and inhibitory receptors. A) Inhibitory receptors on NK cells interact with MHC class I molecules expressed by healthy self-cells mediating an inhibitory signal. B) Target cells that have downregulated MHC class I expression due to stress and upregulated expression of an activating ligand trigger NK cell-mediated killing. C) Target cells express MHC class I molecules, however, when stress ligands are upregulated by target cells they trigger NK cell cytotoxicity because the activation signal overrides the inhibitory signal. Inhibitory signal (–ve), activation signal (+ve) (Vivier et al., 2012). License obtained from Nature Publishing Group; 3397691457574.

1.2.5.1 Inhibitory receptors

NK cells express a repertoire of inhibitory receptors that regulate their activation. Some of these receptors belong to the Ig superfamily such as killer immunoglobulin-like receptors (KIR) that bind to MHC class I, while others are C-type lectin receptors (CD94/NKG2A) that recognise human leucocyte antigen (HLA)-E. Although the structure of these receptors varies, they all share a common motif, the immune-receptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain. The engagement of inhibitory receptors with their ligands leads to the phosphorylation of the ITIM motif and production of phosphatases that prevent Ca^{2+} influx, shutting down NK cell effector function (Ravetch and Lanier, 2000). Thus, inhibitory receptors play substantial roles in NK cell self-tolerance and regulation of NK cell activation. NK cells express a minimum of one inhibitory receptor, specific for self-MHC molecules in order to avoid killing of healthy self-cells. Table 1.2 summarises NK cell inhibitory receptors and their HLA-based cognate ligands.

Table 1.2 Human inhibitory receptors expressed by NK cells and their cognate ligands

Inhibitory receptor (s)	Ligand (s)
CD94/NKG2A	HLA-E
KIR2DL1	HLA-C group 2 alleles (C*02, C*04, C*05, C*06)
KIR2DL2	HLA-C group 1 alleles (C*01, C*03, C*07, C*08)
KIR2DL3	HLA-C group 1 alleles (C*01, C*03, C*07, C*08)
KIR3DL1	Some HLA-A and B alleles expressing Bw4 epitope
KIR3DL2	Some HLA-A alleles (A*03, A*11)
KIR3DL3	Unknown
KIR2DL4	HLA-G
KIR2DL5	Unknown

1.2.5.1.1 Killer immunoglobulin-like receptors

NK cells and a subset of T cells express KIRs, receptors that recognise MHC class I molecules (Moretta and Moretta, 2004). This interaction mediates NK cell tolerance to self-healthy cells, whereas cells that have downregulated MHC class I due to transformation or viral infection become susceptible targets of NK cells. The KIR ligands, HLA-A, HLA-B and HLA-C, are expressed abundantly by nucleated cells (Lanier, 1998). Inhibitory KIRs are characterised by a long cytoplasmic tail (KIR-L)

containing an ITIM. The extracellular Ig domain of inhibitory KIRs confers specificity to different HLA class I alleles. Accordingly, KIRs are divided into two major groups; KIR2DL recognising HLA-C alleles and KIR3DL recognising HLA-A and HLA-B alleles (Thielens et al., 2012).

HLA-C alleles are polymorphic and bind to different types of KIR receptors. Accordingly, HLA-C alleles are classified into two groups: the C2 group (C*02, C*04, C*05, C*06), which bind to KIR2DL1 and the C1 group (C*01, C*03, C*07, C*08), which bind to KIR2DL2/3. Nonetheless, KIR2DL2/3 receptors recognise some HLA-C alleles belonging to the C2 group including C*05:01 and C*02:02 as well as some HLA-B alleles (B*46:01 and B*73:01). On the other hand, KIR3DL1 and KIR3DL2 do not recognise any HLA-C alleles. Instead, these inhibitory receptors recognise HLA-A alleles (Thielens et al., 2012).

KIR expression occurs at the latest stage of NK cell differentiation and correlates with fully functional NK cells. A role for KIRs and their ligands in the context of unrelated haploidentical haematopoietic stem cell transplantation (HSCT) and NK cell-based therapy has been suggested. Ruggeri and colleagues proposed that KIR-mismatched donor NK cells are effective in destroying leukaemic blasts (Ruggeri et al., 2002, Ruggeri et al., 2005). In this context, NK cell killing of autologous cells is inhibited by KIR, as they interact with self-MHC class I alleles expressed by targets. On the other hand, a mismatch between donor allogeneic NK cells and recipient MHC class I alleles triggers NK cell alloreactivity. Thus, alloreactive NK cells are able to destroy residual leukaemic blasts improving the outcome of transplantation (Ruggeri et al., 2005).

1.2.5.1.2 CD94/NKGA

NKG2A is an inhibitory receptor expressed by NK cells as a heterodimer with CD94. This lectin-like inhibitory receptor interacts only with HLA-E, a non-classical MHC class I molecule that has restricted sequence variability. Assembling of HLA-E molecules occurs in the endoplasmic reticulum (ER) using signal peptides of the classical MHC molecules; HLA-A, B and C (Braud et al., 1998). The expression of HLA-E on the cell surface requires association with MHC class I leader-derived peptides and monitors classical MHC class I expression by an indirect mechanism.

Hence, if the expression of MHC class I is downregulated, the expression of HLA-E decreases accordingly. Therefore, NKG2A interaction with HLA-E regulates NK cell functions when MHC class I is dysregulated due to infection or transformation (Kaiser et al., 2005).

1.2.5.1.3 Other inhibitory receptors

NK cell effector function is also regulated by another group of Ig superfamily receptors known as the Ig-like transcripts (ILTs) or leukocyte Ig-like receptors (LIRs). Among these receptors, ILT-2 or LIR-1 contains four typical ITIMs in its cytoplasmic domain that mediate inhibition of NK cell functions. Although these receptors are homologous to KIRs, they display low affinity binding to HLA class I molecules (HLA-A, B and C). LIR1, however, interacts with non-classical HLA-G molecules with better specificity (Vitale et al., 1999).

NK cell inhibition can be mediated independently of MHC class I based-inhibition. Carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 has been found to be upregulated on NK cells in melanoma patients (Stern et al., 2005). This protein contains ITIM that inhibit NK cell cytotoxicity upon interaction with carcinoembryonic antigens on target cells (Stern et al., 2005). In addition, NK cells express killer cell lectin-like receptor G1 (KLRG1) that also bears an ITIM and regulate NK cell functions (Ito et al., 2006). KLRG1 is expressed by 30-50 % of NK cells and is upregulated as a result of NK cell activation and viral infection. Moreover, upregulation of KLRG1 expression by NK cells correlates with NK cell maturation and homeostasis (Huntington et al., 2007). Furthermore, a subset of NK cells express the NK cell receptor protein 1A (CD161) that binds to Lectin-like transcript-1 (LLT1) (Aldemir et al., 2005). LLT1 ligand has been found to be expressed by activated DCs and its interaction with CD161 on NK cells inhibits NK cell cytotoxicity and cytokine production (Rosen et al., 2008).

1.2.5.2 Activating receptors

At early maturation stages, NK cells acquire the expression of a variety of natural cytotoxicity receptors (NCRs). The engagement of these receptors results in positive stimulation of NK cells and direct lysis of target cells (Moretta et al., 2000).

Furthermore, NK cells express other activating receptors including NKG2D, DNAM-1, 2B4 and NKp80 that can directly mediate killing or act as co-receptors (Fig 1.5) (Moretta et al., 2000).

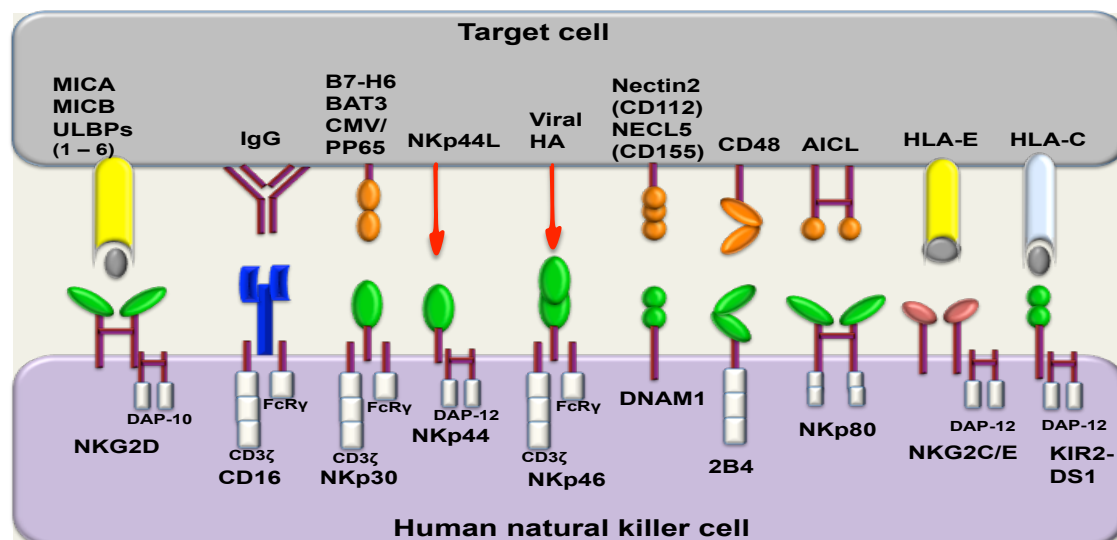


Figure 1.5 Interactions of NK cell-activating receptors with different ligands expressed by target cells.

1.2.5.2.1 Natural cytotoxicity receptors (NCRs)

NCRs are a group of NK cell receptors that mediate direct killing of stressed cells. There are three identified NCRs: NKp30, NKp44 and NKp46. NCRs are germ-line encoded transmembrane receptors that belong to the Ig superfamily. Yet, they are dissimilar in structure and amino-acid sequence.

1.2.5.2.1.1 NKp30

NKp30 is characterised by a V-type single extracellular domain. The NKp30 transmembrane domain has an arginine residue that is attached to an ITAM containing CD3 ζ and Fc ϵ R γ adaptors (Pende et al., 1999). This receptor is constitutively expressed by both resting or cytokine-activated NK cells (Moretta et al., 2000) and plays a substantial role in NK cell interaction with DCs. NK cell recognition of infected DCs occurs via NKp30, a process that leads to NK cell expansion, cytokine production and priming of the adaptive immune response (Ferlazzo et al., 2002). Different cellular ligands for NKp30 have been identified including HLA-B-associated-transcript 3 (BAT3) (Pogge von Strandmann et al., 2007) and the cytomegalovirus (CMV) pp65

protein (Arnon et al., 2005). However, these are not considered as tumour induced ligands as their expression is induced by heat-shock or infection, respectively. The only tumour related stress ligand identified for NKp30 is a member of the B7 family, B7-H6 (Brandt et al., 2009). This ligand is undetectable on healthy tissues and is not induced by stress such as heat-shock or toxicity. In contrast, B7-H6 is broadly expressed by tumour cells including B and T cell lymphomas, carcinomas, melanomas and myeloid leukaemia (Brandt et al., 2009).

1.2.5.2.1.2 NKp44

The second NCR in this group is NKp44 or NCR2. Molecular analysis of NKp44 showed that this receptor has a single extracellular domain displayed as a V-shape. The transmembrane domain of NKp44 comprises a lysine residue that together with the ITAM-containing DAP12 (12 kD signal-transducing adapter) mediate signal transduction (Cantoni et al., 1999). Early studies showed that IL-2 activation induces lysis of different targets resistant to killing by resting NK cells (Grimm et al., 1982, Trinchieri, 1989). Later, it was shown that NKp44 is exclusively expressed by activated NK cells (Vitale et al., 1998). Thus, upregulation of NKp44 by activated NK cells mediates additional binding to stress ligands expressed by target cells, enhancing NK cell killing capacity accordingly (Moretta et al., 2000). In addition, certain type of cells express NKp44 including decidual NK cells (Hanna et al., 2006), mucosal-associated lymphoid tissue NK-22 cells (Cella et al., 2010), that are now classified as ILC3 (Vosshenrich and Di Santo, 2013), and a subset of IFN- γ -producing cells in the tonsils (Ferlazzo et al., 2004). NKp44 masking using mAbs results in partial inhibition of NK cell cytotoxicity against MHC class I-negative tumour cells (Vitale et al., 1998). NKp44 was shown to induce cytotoxicity cooperatively with NKp46 as blocking both receptors led to increased inhibition of NK cell cytolytic function (Moretta et al., 2000). The identification of a stress-induced ligand for NKp44 has been a challenge for a long time. In HIV patients, NKp44 is over-expressed by NK cells. Interestingly, a cellular ligand for NKp44 was found to be expressed on HIV infected CD4 T cells mediating NK cell killing (Vieillard et al., 2005). However, isolation and identification of the NKp44 ligand (NKp44L) was only achieved recently by Baychelier and colleagues (Baychelier et al., 2013). NKp44L is a variant of mixed-lineage leukemia-5 (MLL5) that is strikingly expressed by tumour or stressed cells but not found on healthy cells.

This ligand is highly conserved and contains a C-terminal-motif in its outer domain that interacts specifically with NKp44 (Baychelier et al., 2013). Yet, this does not exclude the existence of other undetected ligands for NKp44.

1.2.5.2.1.3 NKp46

NKp46 (in human) or NCR1 (in mouse) contains C2-type Ig-like domains in its outer membrane whereas the intracellular domain contains ITAM-bearing polypeptide adapters (CD3 ζ and Fc ϵ R γ) that mediate signal transduction. This receptor is expressed by both resting and activated NK cells triggering NK cell cytotoxicity and cytokine production (Moretta et al., 2000). NK cells express NKp46 at high density (NKp46^{bright}), however it was shown that some individuals have NK cells that exhibit a NKp46^{dim} population constituting up to 90 % of the total NK cell population (Sivori et al., 1999). This was associated with low cytotoxicity against NK cell-sensitive-tumour targets by both freshly isolated NK cells or clones derived from NKp46^{dim} individuals suggesting a major role of this receptor in eliminating stressed cells (Sivori et al., 1999). Masking this receptor using anti-NKp46 mAbs resulted in inhibition of NK cell-mediated killing of a variety of human tumours including melanoma, breast carcinoma, liver carcinoma and Epstein Barr virus transformed cell lines. NKp46 was shown to be sufficient to trigger NK cell cytotoxicity against murine tumour cells such as Bw-1502 and YAC-cells (Sivori et al., 1999). Human NKp46 is the only activating receptor that is able to recognise ligands expressed on murine cells indicating that NKp46 ligand is conserved between the two species (Biassoni et al., 1999, Moretta et al., 2000). NKp46 recognises influenza virus haemagglutinin (HA), yet no specific tumour ligand for this receptor has been identified (Arnon et al., 2004). Interestingly, NCR1 knockout mice revealed a central role of NKp46 in preventing the metastasis of B16 melanoma and Lewis-lung carcinoma tumours (Glasner et al., 2012). Using the *Neo* mouse model, Narni-Mancinelli demonstrated a regulatory role of NKp46 including downregulation of NK cell activity and development of T cell responses (Narni-Mancinelli et al., 2012). These mutant mice showed enhanced resistance to viral and bacterial infections associated with hyper-responsiveness of NK cells compared to NK cells from WT mice. Genome analysis revealed a mutation of *Ncr1* genes that encode NKp46 where *Neo* mice have no detectable surface expression of NKp46. Interestingly, *in vivo* blockade of NCR1 in WT mice using mAbs enhanced NK cell activity against YAC-1 tumour cells

suggesting this could be a possible immunotherapeutic tool to augment NK cell activity in the absence of T cells, particularly after HSCT.

1.2.5.2.2 Activating receptors belonging to the CD94/NKG2 family

The NK cell group 2 (NKG2) family is a group of C-type lectin-like receptors comprising CD94/NKG2C and CD94/NKG2E that are specific for HLA-E. These receptors are expressed as heterodimers coupled with CD94, which is a membrane glycoprotein. CD94/NKG2C and E signal via DAP12 signalling molecules and both have been implicated in viral immunity (Braud et al., 1998, Fang et al., 2011). The expansion of NK cell subsets preferentially expressing CD94/NKG2C was observed as a result of specific viral infections including CMV, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) (Béziat et al., 2012). In addition, a distinct subset of NK cells exist in CMV infected individuals that display high levels of CD94/NKG2C expression together with CD57 expression (Lopez-Vergès et al., 2011). In HSCT settings, reactivation of CMV promotes the expansion of a CD94/NKG2C⁺ NK cell subset that is associated with a mature NK cell phenotype and enhanced IFN- γ production (Foley et al., 2012). Hence, the selective expansion of NKG2C⁺ NK cells as a result of viral infection, as well as other associated factors including their mature phenotype and extended life-span, are suggestive of a memory-like function by NK cells (Min-Oo et al., 2013).

Another important receptor that belongs to the NKG2 family is NKG2D. NKG2D is a transmembrane receptor that is expressed as a homodimer. NKG2D signals via the signalling molecule DAP10 (Wu et al., 1999). This receptor is expressed by almost all NK cells, as well as by $\gamma\delta$ T cells and CD8 T cells. NKG2D binds to MHC class I-related chain A/B molecules (MICA/MICB) and UL-16 binding proteins (ULBPs), which are stress-induced molecules that are expressed by tumour cells and viral infected cells to initiate NK cell cytolytic activity (Bauer et al., 1999, Cosman et al., 2001). Remarkably, NK cell activation by NKG2D overrides the inhibition mediated by KIR because the signalling cascade for DAP10 is distinct and not affected by SHP, which is a component of ITIM bearing inhibitory receptors (Watzl, 2003). In mice, the interaction between NKG2D and NKG2D ligands (RAE-1 or H-60), which are expressed by several murine tumour cell lines, results in rejection of cancerous cells

alongside the priming of CTL responses (Cerwenka et al., 2001). Ligands for NKG2D are constitutively expressed in many tumours of epithelial origin including lung, breast, kidney, and colon carcinoma (Groh et al., 1999, Jinushi et al., 2003). In leukaemia patients, Salih *et al.* reported elevated levels of soluble NKG2D ligands which impair NKG2D-mediated killing in the sera of patients, including MICA and MICB (Salih et al., 2003). Overall, recognition of these ligands by effector cells enhances tumour immune-surveillance against cancerous cells (Raulet, 2003).

1.2.5.2.3 DNAX accessory-molecule type-1 (DNAM-1)

DNAM-1 (CD226) is a receptor that belongs to the Ig superfamily of receptors, which comprise two Ig V-type external domains. Besides NK cells, this receptor is also expressed by a variety of cell types including T cells, a subset of B cells, monocytes and platelets. Ligands for DNAM-1 are Nectin2 (CD155) and PVR (CD112) both of which are expressed by tumour cell lines of neuronal and epithelia origin (Pende et al., 2005). Masking DNAM-1 with mAbs results in inhibition of NK cell-mediated cytotoxic function against target cells expressing Nectin2 or PVR ligands, thereby confirming the central role of this receptor in tumour-mediated lysis by NK cells (Pende et al., 2005). In addition, the association of DNAM-1 with lymphocyte function associated antigen-1 (LFA-1) triggers NK cell cytotoxicity and cytokine secretion (Shibuya et al., 1999). The signal transduction pathway used by this receptor remains to be defined.

1.2.5.2.4 2B4

2B4 (CD224) is a member of the signalling lymphocyte activation molecule (SLAM) family. 2B4 is expressed by NK cells and memory CD8⁺ T cells and binds to CD48 which is expressed on lymphocytes and myeloid cells with high affinity (Brown et al., 1998). 2B4 also acts as a co-receptor that induces cytotoxicity and IFN- γ production by NK cells (Nakajima et al., 1999, Kubin et al., 1999). Generally, 2B4 is an activating receptor in humans and an inhibitory receptor in mice. In this context, it has been shown that 2B4-CD48 interactions may result in inhibition of tumour cell lysis. Hence, 2B4 may mediate a regulatory role of NK cells in MHC class I-deficient hosts, or for cells that have downregulated self-MHC expression (McNerney et al., 2005). CD48 has been found to be upregulated by lymphocytes and myeloid cells in response to EBV or

stimulation with mitogens and IFNs (Parolini et al., 2000, Tissot et al., 1997). 2B4 bears cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSM; TxYxxV/I) in its cytoplasmic domain, which play an important role in modifying the function of this receptor, which can be both activating and inhibitory. ITSM binds SH2 domains containing either activating or inhibitory molecules (SHP-1, SHP-2, SAP, and EAT-2). In particular, SAP is implicated in dictating 2B4 signalling (Endt et al., 2007). Immature NK cells in humans and mice lack the expression of SAP transcript. In this context, 2B4 has been shown to play an inhibitory role during early stages of NK cell development mediating self-tolerance before NK cells become fully competent (Sivori et al., 2002).

1.2.5.2.5 NKp80

Almost all NK cells, a subset of $\gamma\delta$ T cells and a subset of CD8 T cells express NKp80. NKp80 recognises a receptor expressed by myeloid cells named activation-induced C-type lectin (AICL). NKp80 interaction with this receptor results in triggering of NK cell cytotoxic functions. Unlike other ITAM-bearing activating receptors, the NKp80 signalling pathway has been shown to be distinct. Using an IL-2-dependent cell line, it was recently found that NKp80 signals via an atypical hemi-ITAM activating the Syk-kinase pathway (Dennehy et al., 2011).

1.2.5.2.6 Activating killer immunoglobulin receptors

KIR genes are complex and highly polymorphic combining *KIR-L* (long cytoplasmic domain) and *KIR-S* (short cytoplasmic domain) genes that both display similarities in their extracellular domains. *KIR-S* receptors, however, have short cytoplasmic tails that do not contain ITIM. In addition, the *KIR-S* transmembrane region contains charged residues that attach to the DAP12 signalling molecule to transmit the activating signal accordingly (Thielens et al., 2012). Although KIR2DS1 has been found to interact with HLA-C2, there is no evidence of interaction between KIR2DS2 with C1 molecules (Pende et al., 2009).

1.2.5.2.7 CD16

NK cells have abundant expression of the Fc receptor CD16 (FcγRIII). CD16 recognises antibody-bound stressed cells via the Fc portion of IgG initiating ADCC, a pathway that induces apoptosis of target cells (Lanier et al., 1991). CD16 is expressed abundantly by CD56^{dim} NK cells. Activation of NK cells via CD16 is very potent and CD16 is the only NK cell activating receptor which is able to trigger NK cell cytotoxicity on its own (Bryceson et al., 2006).

1.2.5.2.8 Other activating receptors

NK cells constitutively express CD2, which is thought to act as an adhesion molecule. However, there is evidence to suggest that CD2 also acts as a co-stimulatory molecule (Inoue et al., 2002). CD69 is another receptor expressed by NK cells upon activation and its expression enhances effector functions of NK cells including, cytotoxicity, proliferation, upregulation of adhesion molecules and cytokine production (Borrego et al., 1999). The CD69 ligand has not yet been identified.

1.2.6 Receptors that mediate NK cell trafficking

NK cells express different chemokine receptors, integrins, adhesion molecules as well as proteins such as sphingosine 1-phosphate (S1P) receptors that regulate their trafficking to different sites. As part of their role in immune surveillance, NK cells are distributed throughout the body. NK cell trafficking is very important for NK cell-based therapies, as NK cells need to traffic to tumour sites in order to deliver meaningful clinical outcomes.

1.2.6.1 Adhesion molecules

NK cells express CD2, CD44, CD54 (ICAM-1), CD56, CD58 as well as different integrins. Integrins are a large family of molecules expressed on the leukocyte cell surface as heterodimers of non-covalently attached α and β subunits. Based on their β subunit, integrins are divided into different subfamilies. The β 1 and β 3 (cytoadhesion) subunits act as extracellular matrix receptors, whereas the β 2 (Leu-CAM) subunit supports cell-to-cell adhesion (Patarroyo et al., 1990). NK cells express α 4 β 1 that interacts with VCAM-1, α 5 β 1 that interacts with fibronectin and α 6 β 1 that interacts

with laminin. The second subfamily of integrins, $\beta 2$, plays a critical role in NK cell signalling and formation of conjugates with target cells (Morris and Ley, 2004). In particular, LFA-1 (CD11a/18) is a key receptor in endothelial cell binding and for the formation of the immunological synapse (Orange, 2008). Moreover, LFA-1 acts as a co-receptor stimulating NK cell functions (Shibuya et al., 1999).

Another crucial component for NK cell migratory function is L-selectin (CD62L). L-selectin is an adhesion molecule which facilitates extravasation of NK cells into high endothelial venules in lymph nodes (LN). This molecule is expressed at differential levels among NK cell populations. CD56^{bright} NK cells express high levels of L-selectin, whereas CD56^{dim} NK cells display lower and variable expression among individuals (Mäenpää et al., 1993, Uksila et al., 1997). Interestingly, it was shown that a unique subset of CD56^{dim}L-selectin⁺ NK cells that exhibit high production of IFN- γ , high proliferative capacity as well as enhanced cytotoxicity compared to CD56^{dim}L-selectin⁻ NK cells (Juelke et al., 2010). L-selectin expression is associated with NK cell migration to the LN, in which NK cells are able to prime adaptive responses by releasing IFN- γ and interacting with resident LN DCs (Chen et al., 2005). L-selectin expression is upregulated upon stimulation of NK cells with IL-12 or IFN- α , whereas stimulation with IL-2 or IL-15 downregulates L-selectin expression by NK cells (Frey et al., 1998).

1.2.6.2 C and CX-Chemokine receptors

NK cells develop in the BM; hence, during the early stages of NK cell differentiation NKP and iNK cells express high levels of CXCR4 in response to CXCL12 (SDF1- α) expressed by the BM parenchyma. As a result, iNK cells are retained in the BM in order to complete their maturation (Mayol et al., 2011). Mobilisation of NK cells from the BM is assisted by CCR1 which mediates downregulation of CXCR4 expression by mature NK cells (Bernardini et al., 2008). However, NK cells display homogenous CXCR4 expression and other factors such as S1P₅ also contribute to NK cell egress from the BM (Walzer et al., 2007). Less mature NK cells express low levels of CX3CR1 and are located at the BM parenchyma, whereas terminally differentiated NK cells exhibit high density of CX3CR1 and are located in BM sinusoids. The acquisition of high CX3CR1 expression supports NK cell egress from the BM and entry into the

circulation. In addition, S1P₅ expression increases on mature NK cells mediating their entry into the blood stream (Mayol et al., 2011).

Chemokine receptor expression varies among NK cell subsets. The majority of circulating mature NK cells express additional receptors including CXCR1, Chemrin23 (Chem23), which along with CX3CR1 support NK cell recruitment towards inflamed tissues via IL-8 and soluble fractalkines. Conversely, CD56^{bright} NK cells display low levels of CX3CR1 expression and do not express CXCR1 (Robertson, 2002). Instead, CD56^{bright} NK cells express CCR7 that is associated with migration to secondary lymphoid tissues in response to CCL19 and CCL21 expressed in the T cell zone. In addition, CD56^{bright} NK cells have high CXCR3 expression, which is a chemokine receptor that mediates binding to CXCL10 and CXCL11 (Campbell et al., 2001). This chemokine receptor is implicated in trafficking of NK cells to LN as well as tumour infiltration as NK cells from *Cxcr3*^{-/-} knock-out mice display poor capacity to accumulate at tumour sites (Wendel et al., 2008).

Chemokine receptor expression by NK cells is altered upon inflammation during infection or in autoimmune disorders (Peng and Tian, 2013). This is associated with the upregulation of chemokine ligands in the disease state that are not typically expressed at steady state. For example, CCR5 is highly expressed by NK cells in rheumatoid arthritis patients and its ligands (CCL3 and CCL5) were found to be upregulated accordingly in inflamed joints (Mack et al., 1999).

In vitro, stimulation with cytokines modifies chemokine expression by NK cells with some receptors being upregulated and some downregulated. IL-2 stimulation upregulates the expression of CCR1, CCR2, CCR4, CCR5 and CCR8 by NK cells (Inngjerdingen et al., 2001). Furthermore, NK cell activation with IL-18 induces the expression of CCR7 (Mailliard et al., 2005). On the other hand, IL-15 stimulation downregulates CX3CR1 expression (Barlic et al., 2003) and IL-12 stimulation downregulates CXCR3 expression by NK cells (Hodge et al., 2002).

A newly described subset of liver-resident NK cells exhibiting features of immunological memory has been characterised by high levels of CXCR6 expression (Paust et al., 2010). The ligand for this chemokine receptor (CXCL16) is secreted by

the liver sinusoids. Interestingly, the maintenance of natural killer T cells (NKT cells), which are innate immune cells enriched within the liver, depends on the interaction of CXCR6 with CXCL16 (Geissmann et al., 2005). Thus, it is likely that those cells derived from liver haematopoietic progenitors actually have a unique chemokine receptor repertoire ensuring the retention of these cells within the liver (Peng and Tian, 2013). Table 1.3 summarises the expression of some chemokine receptors by NK cells and their effect *in vivo*.

Table 1.3 Chemokine receptors expressed by NK cells and their cognate ligands

Chemokine receptor (s)	CD56 ^{bright} NK cells	CD56 ^{dim} NK cells	Ligand (s)	Effect (s)	Reference (s)
CCR1	High	Low	CCL3 and CCL5	Egress from the BM	(Bernardini et al., 2008)
CCR2	Negative	Negative	CCL2 and CCL7	Recruitment of NK cells to the liver during CMV infection	(Hokeness et al., 2005)
CCR4	Negative	Variable	CCL17, CCL22	NK cell responses against mycobacterial infections	(Stolberg et al., 2011)
CCR5	High	Negative	CCL3, CCL4 and CCL5	Accumulation of NK cells during viral infections and autoimmune diseases.	(Grégoire et al., 2007, Grégoire et al., 2008)
CCR7	High	Negative	CCL19, CCL20 and CCL21	Migration to LN	(Mailliard et al., 2005, Marcenaro et al., 2009)
CX3CR1	Low	High	CX3CL1	Migration to non-lymphoid inflamed tissues	(Barlic et al., 2003)
CXCR1	Negative	High	IL-8	Migration to non-lymphoid inflamed tissues	(Inngjerdingen et al., 2001, Parolini et al., 2007)
CXCR3	High	Low	CXCL9, CXCL10 and CXCL11	Migration to LN, tumour infiltration	(Martín-Fontecha et al., 2004)
CXCR4	High	High	CXCL12	Retention within BM	(Mayol et al., 2011)
CXCR6	Variable	Negative	CXCL16	Liver NK cells (memory function?)	(Paust et al., 2010)

1.2.7 NK cell effector function

Upon recognition of stressed cells, adhesion molecules play an indispensable role in initiating interactions between NK cells with their targets to form what is known as a

lytic immunological synapse (IS). NK cells store cytotoxic mediators in granules within lysosomes whereby they can effectively kill target cells by exocytosis. In addition, NK cells kill their targets by other mechanisms including death-receptor and ADCC pathways and are able to prime other immune responses via secretory cytokines.

1.2.7.1 NK cell killing via the perforin-dependent pathway (exocytosis)

Exocytosis is the main mechanism that NK cells utilise to kill targets. NK cells from perforin-deficient mice show reduced cytotoxicity and fail to lyse some tumorigenic targets (Kagi et al., 1994). Cytolytic granules of cytotoxic cells contain perforin and structurally related proteases (granzymes). Perforin mediates pore formation in the plasma membrane of the target cell where granzymes are delivered in a calcium-dependent manner and diffused into the target cell. Granzyme B is the most powerful activator of caspase-dependent and caspase-independent cell death, cleaving target cell proteins at multiple and specific residues (Trapani and Smyth, 2002). In contrast, granzyme A cannot activate the caspase pathway, but directly can cleave nuclear proteins (Beresford et al., 1999).

Granzyme B diffusion into target cells is partially mediated by cation-independent mannose 6-phosphate receptor in an endocytosis-dependent manner. Granzyme B activates caspases or the pro-apoptotic member of the Bcl-2 family (BH3-interacting domain death agonist) to result in apoptosis (Thomas et al., 2001). Interestingly, cytotoxic cells contain a lysosomal protease (cathepsin B) within their cytotoxic granules that inactivates perforin supporting its diffusion back to the cytotoxic cell membrane after degranulation is complete (Balaji et al., 2002). This pathway is potent, resulting in rapid killing of viral-infected and tumour-transformed cells within minutes.

NK cell cytotoxic function relies on the ability of transporting lytic granules to the IS. During degranulation, the lysosome associated membrane protein (CD107a) is transported to the surface of NK cells facilitating the mobility of the lytic granules (Krzewski et al., 2013).

1.2.7.2 NK cell mediated cytotoxicity via death receptor pathways

NK cells can also induce death of target cells in a death receptor-dependent manner. NK cells express receptors belonging to the TNF family, including FasL and TRAIL. Interaction of these receptors with their ligands results in apoptosis (Smyth et al., 2005).

The majority of NK cells and CTLs constitutively express FasL. Interaction of Fas with its ligand, FasL, leads to the clustering of death domains (DD) on target cells. Subsequently, DD binds caspase-8 activating effector caspases that are committed to cellular apoptosis (Ashkenazi and Dixit, 1998). Although many tumour types do not express Fas, NK cells contribute to the induction of Fas expression by stressed target cells via the secretion of IFN- γ . Thus, Fas-FasL interaction correlates with tumour regression *in vivo* and is an important pathway in cancer clearance (Screpanti et al., 2001).

In addition, NK cells express TRAIL, which is a type II membrane protein. TRAIL plays a central role in eliminating TRAIL-sensitive tumour cell lines such as fibrosarcoma, renal adenocarcinoma and B-cell hybridoma in an IFN- γ dependent manner (Takeda et al., 2001b). Many ligands for TRAIL have been described to which two of them can effectively induce apoptotic signals; TRAIL-R1 (DR4) and TRAIL-R2 (DR5). NK cell killing of TRAIL-sensitive targets is caspase-dependent by clustering of DD (Ashkenazi and Dixit, 1998). The anti-metastatic effect of NK cells has been found to be partially mediated by TRAIL (Takeda et al., 2001a). Furthermore, TRAIL is also implicated in anti-viral activity as the secretion of IFN- α or β upregulates TRAIL expression by NK cells as well as increasing the expression of TRAIL ligand by virally infected cells (Sato et al., 2001). Similarly, *in vitro* activation of NK cells by cytokines such as IL-2 or IL-15 results in upregulation of TRAIL expression (Smyth et al., 2005).

1.2.7.3 Production of inflammatory cytokines by NK cells

NK cells participate in a variety of responses via the production of inflammatory and immunoregulatory cytokines. Typically, CD56^{bright} NK cells produce inflammatory cytokines more abundantly compared to CD56^{dim} NK cells. The type of cytokines produced by NK cells in early inflammatory events elicits different responses against particular stimuli.

IFN- γ production by NK cells is induced by cytokines produced by macrophages and DCs, typically IL-12 and IL-18. In addition, IFN- α , a cytokine that is abundantly produced during viral infection, synergises with IL-12 or IL-18 to promote IFN- γ production (Matikainen et al., 2001). TNF- α is another cytokine that mediates an additive effect with IFN- α by stimulating IFN- γ production by NK cells (Marshall et al., 2006). NK cell recognition of target cells via activating receptors such as NKG2D also induces the production of IFN- γ . Secreted IFN- γ by NK cells mediates critical roles in anti-microbial and anti-tumour responses including: (i) triggering the transition from the innate to the adaptive immune response by priming T helper cells; (ii) stimulation of B cell isotype switch; (iii) promoting the interaction of leukocytes with epithelial cells; and (iv) upregulation of the expression of adhesion molecules (ICAM-1, VCAM-1) and chemokines (CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5), thereby mediating trafficking of effector cells to sites of inflammation (Schroder et al., 2004). In addition, secretion of IFN- γ by NK cells inhibits tumour angiogenesis (Hayakawa et al., 2002). IFN- γ production is negatively regulated by IL-4, IL-10 and TGF- β (Schindler et al., 2001).

NK cells produce modest levels of TNF- α upon stimulation with cytokines or mitogens (Cooper et al., 2001c). *In vivo*, it is believed that the engagement of NKp30 on NK cells with DCs triggers TNF- α production, which in turn mediates DC maturation and shapes the adaptive immune response accordingly (Vitale et al., 2005). In addition, TNF- α enhances NK cell cytotoxicity by upregulating the adhesion molecule ICAM-1 in a NF- κ B-dependent manner (Wang et al., 2012).

Granulocyte macrophage-colony-stimulating factor (GM-CSF) is abundantly produced by NK cells. This cytokine mediates a critical function, in that it stimulates haematopoietic stem cells (HSC) to rapidly produce granulocytes in order to combat infections. GM-CSF production is enhanced in response to cytokines such as IL-15 or its combination with IL-12 or IL-18 (Cooper et al., 2001c). NK cells under certain stimulating conditions produce other cytokines including IL-5 (Warren et al., 1995, Loza et al., 2002), IL-6 (Hall et al., 2010), IL-8 (Roda et al., 2006), IL-10 and IL-13 (Cooper et al., 2001c, Mehrotra et al., 1998).

1.2.8 Tissue-specific NK cells

1.2.8.1 Liver NK cells

NK cells constitute up to 50 % of total hepatic leukocytes (Norris et al., 1998). Almost equal proportions of CD56^{bright} and CD56^{dim} NK cells exist within the liver. It is believed that liver NK cells are generated from NKP recruited from the BM to the liver via the blood circulation and therein acquire their distinct characteristics and are retained within the liver (Moroso et al., 2011). Liver CD56^{bright} NK cells express significantly higher levels of CD69, NKp44 and HLA-DR, and less L-selectin than PB CD56^{bright} NK cells. In addition, liver CD56^{dim} NK cells express less KIRs and show reduced cytotoxicity compared to PB CD56^{dim} NK cells (Burt et al., 2009). The fact that liver NK cells are less cytotoxic regardless of their activation status indicates that liver NK cells have a regulatory function. In this context, the liver contains a high percentage of NKG2A⁺/Ly49⁻ NK cells that are not responsive to IL-12 and IL-18 stimulation. In addition, liver NK cells are exposed to high levels of IL-10 produced by kupffer cells which dampens the effect of other inflammatory cytokines (Lassen et al., 2010). This indicates a tolerogenic nature of liver-resident NK cells to gut-derived antigens. Indeed, the liver microenvironment is persistently exposed to endotoxins. In addition, the expression of MHC class I by hepatocytes has been shown to be low or absent. Thus, liver NK cells are required to be less aggressive to prevent hyperactivity. However, liver NK cells become potent killers when co-cultured with kupffer cells in the presence of LPS indicating an intrinsic capacity to protect the liver during infection or against tumour cells (Burt et al., 2009). Experiments in mouse showed that two subpopulations of NK cells exist in the liver; DX5⁺CD49⁻ NK cells that are found in afferent and efferent blood and DX5⁻CD49⁺ NK cells that are present within the liver sinusoids. The latter subpopulation is believed to develop distinctly from hepatic haematopoietic progenitors and can mediate memory like functions (Peng et al., 2013).

Liver NK cells influence to the pathogenesis of liver infections such as HBV (Maini and Peppas, 2013) and HCV (Glasner et al., 2012). Liver NK cells can directly kill infected hepatocytes by exocytosis or via the death-receptor pathway. In addition, the production of IFN- γ and TNF- α by liver NK cells mediates T cell responses against infected hepatocytes. However, in chronic infection, liver NK cells promote liver damage as a result of NK cell mediated-lysis of infected hepatocytes or by elimination

of effector T cells. In particular, TRAIL has been found to be upregulated by liver NK cells as a result of liver inflammation, and its ligand (DR5) has been found to be expressed by liver hepatocytes in chronic HBV, thereby mediating apoptosis (Dunn et al., 2007).

1.2.8.2 NK-22 cells

NK-22 cells, recently classified as ILC3 cells (Vosshenrich and Di Santo, 2013), are selectively present at the gut mucosa, Peyer's patches and tonsils. NK-22 cells display poor cytotoxic function and almost no secretion of IFN- γ . As the term indicates, NK-22 cells produce high levels of IL-22, as well as IL-26 and leukaemia inhibitory factor, that both of which are believed to mediate epithelial protection and have an innate anti-inflammatory role (Cella et al., 2009).

1.2.8.3 Thymic NK cells

Thymic NK cells were firstly described by Vosshenrich and colleagues (Vosshenrich et al., 2006). These cells represent a distinct lineage of NK cells expressing high levels of CD56, CD127 (IL-7R α) and the transcription factor GATA-3, and hence classified as ILC2 (Vosshenrich and Di Santo, 2013). Thymic NK cells produce cytokines including IFN- γ , TNF- α and GM-CSF, yet exhibit a lower killing capacity which associated with a lower expression of granzyme B compared to splenic NK cells (Vosshenrich et al., 2006).

1.2.8.4 Uterine NK cells

During pregnancy, more than 40 % of leukocytes in the maternal uterine mucosa (decidua) are CD56^{bright} NK cells. These cells are known as decidual NK (dNK) cells and they mediate an essential role in vascular remodelling and placental growth (Hanna et al., 2006). The interaction between dNK cells and trophoblasts stimulates the production of pro-angiogenic molecules and regulates trophoblast evasion (Moffett-King, 2002). As these cells are specialised in pregnancy-specific activities, dNK cells have poor cytotoxic functions (Hanna et al., 2006).

dNK cells express NKp44 and NKp30. The interaction of these receptors with their ligands that are expressed by stromal decidual cells and trophoblasts induces the production of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), angiopoietins and TGF- β 1, all of which are mediators that promote vascular growth (Moffett-King, 2002, Vacca et al., 2008). In addition, trophoblast cells secrete HLA-G as soluble ligands that bind to KIR2DL4 expressed by dNK cells promoting the induction of pro-angiogenic cytokines. dNK cells have been shown to produce high levels of IL-8 and CXCL10 that regulate the migration of extravillous trophoblast cells towards the decidual basalis (Hanna et al., 2006). This recruitment pattern mediates spiral artery invasion and is critically important for uterine vascular remodelling and placental development (Le Bouteiller, 2013).

1.2.8.5 Cord blood NK cells

NK cells constitute 15 – 30 % of the total CB mononuclear cell (CBMC) population (Kotylo et al., 1990). CD56^{dim} NK cells comprise 90 % of CB NK cells whereas CD56^{bright} constitute 10 % showing similar NK cell subset proportions to PB (Dalle et al., 2005, Tanaka et al., 2003). Studies of CB NK cell function show inconsistencies. It has been reported that CB NK cells have less lytic capabilities than PB NK cells, although CB NK cells were responsive to IL-2 and IL-12 (Gaddy et al., 1995). Additionally, CB contains CD56⁻CD16⁺ cells, a population that is not found in PB (Gaddy et al., 1995). CD56^{dim} CB NK cells express similar percentages of triggering receptors (NKp46 and NKG2D) to PB NK cells (Dalle et al., 2005, Wang et al., 2007). However, Tanaka *et al.* reported that only one-third of CB CD56^{dim} NK cells were able to react against K562 cells, which was attributed to a lower expression of adhesion molecules (CD2, CD11a, CD18, DNAM-1) in comparison to PB NK cells. Dalle *et al.* reported the lack of L-selectin expression by CB NK cells as a contributory factor for their unresponsiveness to K562 cells (Dalle et al., 2005). Finally, other researchers attributed the reduced CB NK cell function to higher expression of the inhibitory receptors NKG2A/CD94, along with a lower expression of granzyme B (Wang et al., 2007).

Our group also conducted a study that aimed to characterise CB NK cells in comparison to PB NK cells (Luevano et al., 2012a). The proportion of CD56^{bright} NK cells was

higher in CB than PB thereby contradicting other reports (Dalle et al., 2005, Tanaka et al., 2003). CB NK cells expressed higher levels of CD94/NKG2A and lower levels of granzyme B, perforin, and Fas-ligand, affecting their killing capacity. CB NK cells expressed lower levels of triggering receptors involved in cancer cell ligation including DNAM-1 and NKp46. Although there was no difference in the percentage of NKG2D expression, mean fluorescence intensity was lower on CB than PB NK cells. CXCR4 was highly expressed by CB NK cells indicating their tendency to home to the BM. CB NK cells degranulated to a level comparable to PB NK cells in response to the K562 cell line, but were not able to kill K562 cells unless activated with IL-2 (Luevano et al., 2012a).

1.2.9 Do NK cells have memory?

Immunological memory is a property of adaptive immune cells in which B and T cells display specific recognition of a particular antigen and a robust response upon secondary exposure. B and T cells undergo receptor rearrangement and retain long-lasting immunity for that antigen. Unlike B and T cells, NK cell recognition relies on a limited number of germ-line encoded activating and inhibitory receptors. In general, NK cell activity is dictated by the signal delivered by inhibitory and/or activating receptors triggered by downregulation of MHC expression or upregulation of activating ligands by stressed targets. However, evidence of NK cell memory-like properties has been recently reported, raising the possibility that NK cells can retain specific memory.

The first example of NK cell memory was demonstrated in a MCMV model by Sun and colleagues (Sun et al., 2009). NK cell-deficient mice display elevated rates of viral replication. MCMV infected cells express the glycoprotein m157, which is a viral MHC class I-like decoy molecule (Adams et al., 2007). In C57BL/6 mice, NK cells express Ly49H, which is an activating receptor that signals via ITAM-bearing DAP12 and the adapter DAP10. Recognition of m157 by Ly49H⁺ NK cells results in activation of signalling components and production of IL-12 by infected DCs, promoting NK cell cytotoxic functions accordingly. Interestingly, Ly49H⁺ NK cells undergo clonal-like expansion, resulting in three times more NK cells in the spleen and ten times more in the liver during the first week of exposure. Thereafter, Ly49H⁺ NK cells undergo a

contraction phase, but a pool of long-lived cells persists for several months. Adoptive transfer of Ly49H⁺ NK cells into a naïve host followed by MCMV challenge results in a robust anti-viral response against m157 glycoproteins suggestive of NK cell immunological memory (Sun et al., 2009). Similarly, many reports have described enhanced and selective expansion of CD94/NKG2C⁺ NK cells during HCMV infection (Gumá et al., 2006, Kuijpers et al., 2008). In this context, a higher frequency of CD94/NKG2C⁺ NK cells were detected in HCMV seropositive individuals whereas the frequency of this subset in uninfected individuals was very low (1 %) (Gumá et al., 2004, Gumá et al., 2006). In addition, NK cells were also shown to “remember” previous exposure to herpes simplex virus- 2 (HSV-2) antigens by displaying higher IFN- γ production as compared to naïve NK cells, which mediated anti-viral protection (Abdul-Careem et al., 2012).

Further evidence of liver-restricted memory NK cells was observed in mice following sensitisation with chemical haptens such as DNFB and oxazolone suggesting an antigen-specific response by NK cells. This model was demonstrated by Von Andrian and colleagues and was based on hapten-induced contact-hypersensitivity (CHS) (O'Leary et al., 2006). It was thought that T cells mediate CHS, however, *Rag*-deficient mice that do not have T cells can develop CHS, which was mediated by NK cells. NK cells exposed to hapten persisted for four weeks after initial exposure and could be restimulated restrictively by the same antigen. Furthermore, DNFB-sensitised donor hepatic NK cells mediate vigorous CHS responses when adoptively transferred into naïve *Rag2*^{-/-}*IL2R γ* ^{-/-} mice (O'Leary et al., 2006). Hapten specific memory was mostly mediated by the Thy-1⁺ and Ly49C/I⁺ NK cell subsets among hepatic NK cells. Yet, it remains unknown why hapten-specific memory NK cells accumulate favourably in the liver. Unlike MCMV memory NK cells, liver restricted memory NK cells do not undergo clonal expansion but display memory features by exhibiting specificity for the sensitising agent. The distribution of these cells resembles invariant NKT (iNKT) cells, whereby the homeostasis and survival of these cells is CXCR6-dependent (Paust et al., 2010). CXCR6 expression by liver restricted memory NK cells allows the cells to respond to CXCL16, which is constitutively expressed by liver sinusoids. However, CXCR6 expression alone is not sufficient to induce memory features of NK cells, as 20 % of liver NK cells are CXCR6⁺ (Geissmann et al., 2005). Thus, CXCR6 expression by liver-restricted memory NK cells is required for their persistence, but not for their

recognition. Furthermore, hepatic NK cells from viral antigen immunised mice (including influenza virus or vesicular stomatitis virus) mediate a sustained delayed hypersensitivity response compared to splenic NK cells exposed to viral infection when adoptively transferred into naïve *Rag2*^{-/-}*IL2Rγc*^{-/-} mice (Paust et al., 2010).

In addition, memory-like features of NK cells could be observed following cytokine stimulation. Cooper and colleagues showed that NK cells activated with IL-12 and IL-18 could be detected up to three weeks after transfer into naïve hosts (Cooper et al., 2009). Interestingly, these NK cells are able to produce higher levels of IFN-γ than unstimulated NK cells when restimulated with IL-15 and IL-12. Thus, cytokine-induced memory-like NK cells display two important characteristics of an adaptive response - they are long-lived and they retain the intrinsic capability to respond differently to restimulation than naïve NK cells. However, cytokine-induced memory-like NK cells have no specificity and do not exhibit enhanced cytotoxicity or proliferation compared to naïve NK cells (Cooper et al., 2009).

1.3 Regulation of NK cell functions by cytokines

NK cell functions are controlled by cytokines. Cytokines are soluble mediators released by innate or adaptive immune cells that play a key role in NK cell development, activation, expansion, trafficking and survival. Different cytokines trigger different NK cell effector functions. NK cells express receptors for several cytokines that confer different binding affinities and responsiveness. In particular, cytokines of the common γ chain (γ_c) family including IL-2, IL-7, IL-15 and IL-21 are implicated in NK cell function. In addition, NK cells effectively respond to cytokines released by activated innate immune cells including IL-12 and IL-18, bridging the innate immune response with the adaptive immune response. Table 1.4 summarises the cytokines known to induce biological effects on NK cells.

Table 1.4 Cytokines that induce biological effects on NK cells

Cytokine (s)	Source (s)	Positive biological effect (s)	Negative biological effect (s)	Reference (s)
IL-2	Activated T cells (T _H 1)	NK cell proliferation Induction of LAK	Expansion of Tregs Induction of AICD	(Barkholt et al., 2009, Ghiringhelli et al., 2005)
IL-7	BM stroma, Fibroblast	NK cell development and homeostasis	No contribution to NK cell cytotoxic function	(Cavazzana-Calvo et al., 1996)
IL-12	DCs and macrophages	Production of IFN- γ and other inflammatory cytokines NK cell cytotoxicity Upregulation of adhesion molecules	Cytokine-induced apoptosis	(Huang et al., 2011, Marcenaro et al., 2005, Smyth et al., 2000)
IL-15	Monocytes, DCs, BM stromal cells, thymus	NK cell development and survival NK cell expansion NK cell cytotoxicity	None	(Liu et al., 2000, Özdemir et al., 2005, Pillet et al., 2009, Ranson et al., 2003)
IL-18	DCs and macrophages	Inflammatory cytokine production Upregulation of chemokine receptors (CCR7)	Cytokine-induced apoptosis	(Agaugue et al., 2008, Bellora et al., 2010, Dinarello, 1999, Mailliard et al., 2005, Huang et al., 2010)
IL-21	Activated T cells (T _H 1), NKT cells	NK cell proliferation NK cell functional maturation NK cell cytotoxicity	Inhibition of IL-15-induced proliferation Induction of immunoregulatory functions (IL-10 production) NKG2D down-regulation	(Brady et al., 2004, Burgess et al., 2006)

1.3.1 Interleukin-2

IL-2 was the first interleukin described and was initially called a T cell growth factor. This cytokine is primarily released by activated T cells and supports T cell differentiation, growth and proliferation. Later on, it was found that IL-2 augments NK cell cytotoxic function both *in vitro* and *in vivo*. Notably, IL-2 deficient individuals develop severe combined immune deficiency (Caligiuri et al., 1993, Henney et al., 1981).

The IL-2 receptor (IL-2R) complex consists of three subunits: IL-2R α , IL-2R β and IL-2R γ . IL-2 binding to IL-2R β and IL-2R γ , which are expressed constitutively by all NK cells, confers intermediate affinity binding (Fig 1.6). However, CD56^{bright} NK cells express the trimeric receptor complex, associated with IL-2R α , that leads to high affinity binding (Caligiuri et al., 1990). Also, it is worth noting that kinetics of association and dissociation of IL-2 to its receptor subunits can vary. IL-2 rapidly associates with IL-2R α subunit within seconds, but dissociates in similar kinetic rates. Whereas the involvement of both receptors, IL-2R α and IL-2R β , confers fast association with IL-2 and slow dissociation, hence provide a stable interaction (Waldmann, 1991). The γ c is a critical component of this receptor, as it is associated with the signal transducing molecule Janus tyrosine-kinase (JAK). Phosphorylation of JAK-1 and JAK-3 initiates different signal transducer and activator transcripts (STAT) pathways. IL-2 mainly phosphorylates STAT5 (STAT5a and STAT5b), but also activates other signalling pathways to a lesser extent including STAT1 and STAT3. STAT5 dimerises upon phosphorylation and diffuses into the nucleus to activate transcription of target genes (Sugamura et al., 1996). It is noteworthy that IL-2R α by itself has low affinity for IL-2 and is devoid of activating signalling components (Becknell and Caligiuri, 2005). IL-2R α is upregulated upon stimulation and is also expressed by regulatory T cells (Tregs). Thus, IL-2 acts as a regulatory cytokine mediating peripheral tolerance, as well as contributing to activation-induced cell death (AICD) in some circumstances (Lenardo, 1996). Defects in IL-2 or IL-2R α genes results in lymphoproliferative and autoimmune disorders (Sadlack et al., 1995, Willerford et al., 1995), whereas IL-2R β deficient mice lack NK cells (Gilmour et al.,

2001, Suzuki et al., 1997). Thus, IL-2 does not appear to be necessary for NK cell development although its intermediate affinity subunit (IL-2R β and IL-2R γ) is strictly required as IL-15 binding to this subunit drives NK cell development.

In vivo, CD56^{bright} NK cells constitutively express the heterotrimer complex of the IL-2 receptor. Thus, low concentrations of IL-2 are sufficient to deliver high affinity binding to CD56^{bright} NK cells and activation of inflammatory cascades. CD56^{bright} NK cells produce relatively high levels of IFN- γ , which further stimulates APCs to produce IL-12 and consequently polarises T cell specific responses (Fehniger et al., 2003). CD56^{dim} NK cells can also respond to IL-2, although they require higher concentrations (Cooper et al., 2001a). Culture of NK cells in the presence of IL-2 leads to upregulation of activation receptors, proliferation and enhanced killing of target cells. Due to the potent effects of IL-2 in immune responses, IL-2 was the first cytokine administered in clinical trials in order to enhance NK cell cytotoxicity against tumour cells (Burns et al., 2003, Caligiuri et al., 1993, Rosenberg et al., 1987).

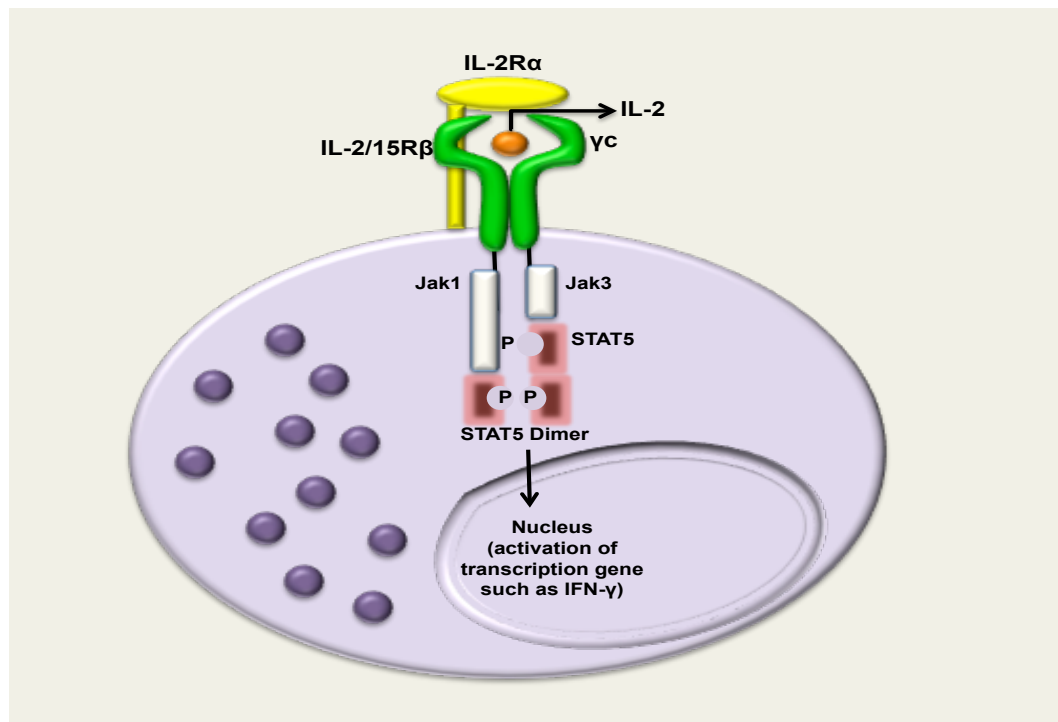


Figure 1.6 IL-2 receptors and STAT5 signalling components. IL-2R α has a very short cytoplasmic tail that is not attached to signalling components whereas IL-2R β and IL-2R γ are capable of signal transduction (Waldmann, 2006). License obtained from Nature Publishing Group; 3505950597219.

1.3.2 Interleukin-7

IL-7 is a γc cytokine that also shares the IL-2/IL-15R β subunit. IL-7 promotes maturation and proliferation of NK cells. The role of this cytokine in NK cell maturation was assessed by experiments that aimed to generate NK cells from CD34⁺ HSC, as it was shown that IL-7 alongside IL-2 and stem cell factor (SCF) mediates maturation of committed NK cells (Cavazzana-Calvo et al., 1996).

1.3.3 Interleukin-12

IL-12 is a heterodimeric cytokine also named NK stimulating factor (NKSF). Initially, it was observed that IL-12 was produced by transformed human B-cell lines and found to stimulate the generation of lymphokine activated killer (LAK) cells. IL-12 is produced by APCs including macrophages and DCs, and it modulates functions of both innate and adaptive immune cells. This cytokine stimulates the transcription and production of IFN- γ that promotes T_H1 cell differentiation. IL-12 also promotes the production of other cytokines such as GM-CSF and TNFs. Moreover, IL-12 enhances NK cell cytotoxic function against many resistant tumour targets (Gately et al., 1994, Trinchieri, 1995).

The IL-12 receptor (IL-12R) consists of two subunits: IL-12R β 1 and IL-12R β 2. NK cells and resting T cells constitutively express IL-12R β 1, whereas IL-12R β 2 expression is induced by cytokines (Fig 1.7) (Presky et al., 1996, Wang et al., 2000). The IL-12R is also expressed by DCs and B cell lines. IL-12 interaction with the IL-12R β 1 and IL-12R β 2 subunits results in activation of several Jak/STAT pathways including STAT1, STAT3 and STAT5 (Presky et al., 1996). Nonetheless, IL-12 primarily activates the STAT4 pathway which appears to be vital for T cell and NK cell functions, as IL-12 activity entirely abrogated in STAT4-deficient mice (Kaplan et al., 1996). Interestingly, activation of the STAT4 signalling pathway by IL-12 leads to the transient activation of NK cells. This results in increased production of ROS and induction of NK cell apoptosis in the case of prolonged stimulation (Huang et al., 2011).

Besides the central role that IL-12 has in NK cell function, IL-12 has also been implicated in NK cell development and differentiation (Loza and Perussia, 2004). IL-12

has been shown to enhance the cytotoxicity of NK cells generated *in vitro*. NK cells differentiated from CD34⁺ CB HSCs secrete significantly higher levels of TNF- α and GM-CSF when stimulated with combinations of IL-12 and IL-15 or IL-21 (Bonanno et al., 2009). This was also confirmed by our group in studies have shown that NK cells generated from CB HSC *in vitro* exhibited an increased cytotoxicity and cytokine secretion after IL-12 stimulation (Luevano et al., 2014). A recent study showed that the addition of low concentrations of IL-12 to *ex vivo* differentiated CB NK cells promotes the expression of L-selectin and KIR, but conversely reduces the expression of CXCR3 (Lehmann et al., 2014). Overall, IL-12 is a powerful enhancer of anti-tumour activities including: (i) NK cell production of inflammatory cytokines, (ii) NK cell killing of tumour targets by the perforin-dependent pathway, (iii) maturation of DCs and priming of adaptive responses accordingly (Agaugue et al., 2008, Smyth et al., 2000). However, systemic administration of IL-12 as an immunotherapeutic drug often results in significant toxicities (Car et al., 1999, Nastala et al., 1994).

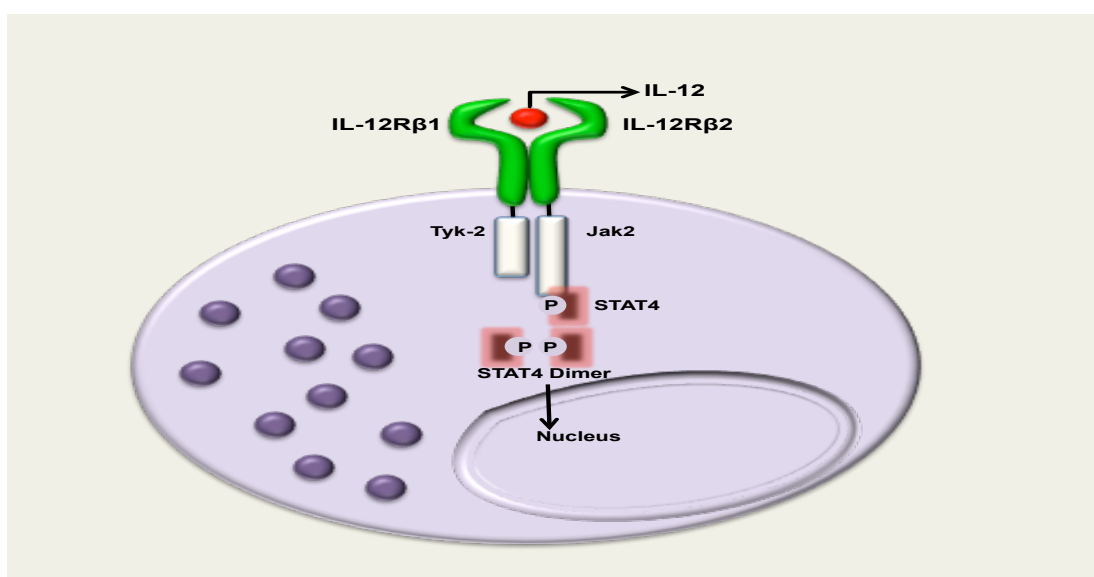


Figure 1.7 Activation of STAT4 pathway by IL-12. IL-12 produced by macrophages and DCs binds to constitutively expressed heterodimer IL-12 receptor on NK cells leading to phosphorylation of Tyk-2 and Jak2 and activation of STAT4 signalling pathway (Trinchieri, 2003). License obtained from Nature Publishing Group; 3505950881267.

1.3.4 Interleukin-15

IL-15 is essential for the main functions of NK cells including development, proliferation, homeostasis and cytotoxicity (Allavena et al., 1997, Evans et al., 1997,

Mrozek et al., 1996, Ranson et al., 2003). IL-15 shares many physiological properties with IL-2 and also binds to the IL-2R β and IL-2R γ subunits to initiate signalling cascades that are similar to those induced by IL-2 (Carson et al., 1994). However, IL-15 is more ubiquitous than IL-2 as many cell types including DCs, macrophages, and thymus and BM stromal cells produce it. As such, IL-15 serves a crucial role in NK cell development and homeostasis. IL-15 possesses its own receptor, IL-15R α , that unlike the IL-2R α confers high affinity binding of IL-15. The IL-15R α mediates several modes of interaction with IL-15. Although it was proven that IL-15 can be trans-presented via IL-15R α on macrophages and DCs to NK cells (Koka et al., 2004), there is evidence that IL-15 could be also presented in “*cis*” by the IL-15R α on NK cells in an autocrine manner (Fig 1.8) (Zanoni et al., 2013). In addition, IL-15R α can form a trimeric receptor complex by association with IL-2/15R β and IL-2/15R γ mediating high affinity binding to IL-15 (Ring et al., 2012).

The similarities between IL-2 and IL-15 raised an important question as to “how two cytokines sharing similar receptors and signalling pathways induce distinctive biological effects?” Indeed, while IL-15 predominantly induces NK cell survival and proliferation by upregulating the anti-apoptotic gene Bcl-2 (Ranson et al., 2003), IL-2 seems to potentiate NK cell activity, but also induces cell death (Waldmann, 2006). The clear difference between the two cytokines is their unique α subunits. The IL-2R α unit has a short cytoplasmic tail (13 residue) and it is not known whether it attaches to any signalling component. In contrast, the IL-15R α subunit contains 43 residues in its cytoplasmic domain and it can recruit other signalling molecules such as the TNF-receptor-associated factor 2 (TRAF2) and Syk (Becknell and Caligiuri, 2005, Bulfone-Paus et al., 1999). In addition, the mechanism of action seems to be different between these two cytokines. *In vitro*, IL-2R β and IL-2R γ are expressed by T cells and the addition of IL-2 results in induction of IL-2R α and IL-2 receptors that are closely associated with each other (Damjanovich et al., 1997). Conversely, IL-15 is presented to NK cells by DCs mainly in a membrane-bound form via the IL-15R α to form an immunological synapse (Dubois et al., 2002). This results in the secretion of IFN and stimulation of nuclear factor- κ B (NF- κ B) (Waldmann, 2006). The IL-15R α /IL-15 complex can be recycled in endosomal vesicles and expressed for many days. Moreover, this complex presents IL-15 to cells expressing IL-2R β and IL-2R γ , but not to those expressing IL-2R α (Dubois et al., 2002).

Due to the potent effect of IL-15 on NK cells, this cytokine has become a potential candidate for NK cell-based therapy. In this regard, IL-15 may mediate many functions; promotion of NK cell effector functions, maintenance of mature NK cells and peripheral homeostasis, as well as the differentiation of NK cells from haematopoietic progenitors in the context of HSCT.

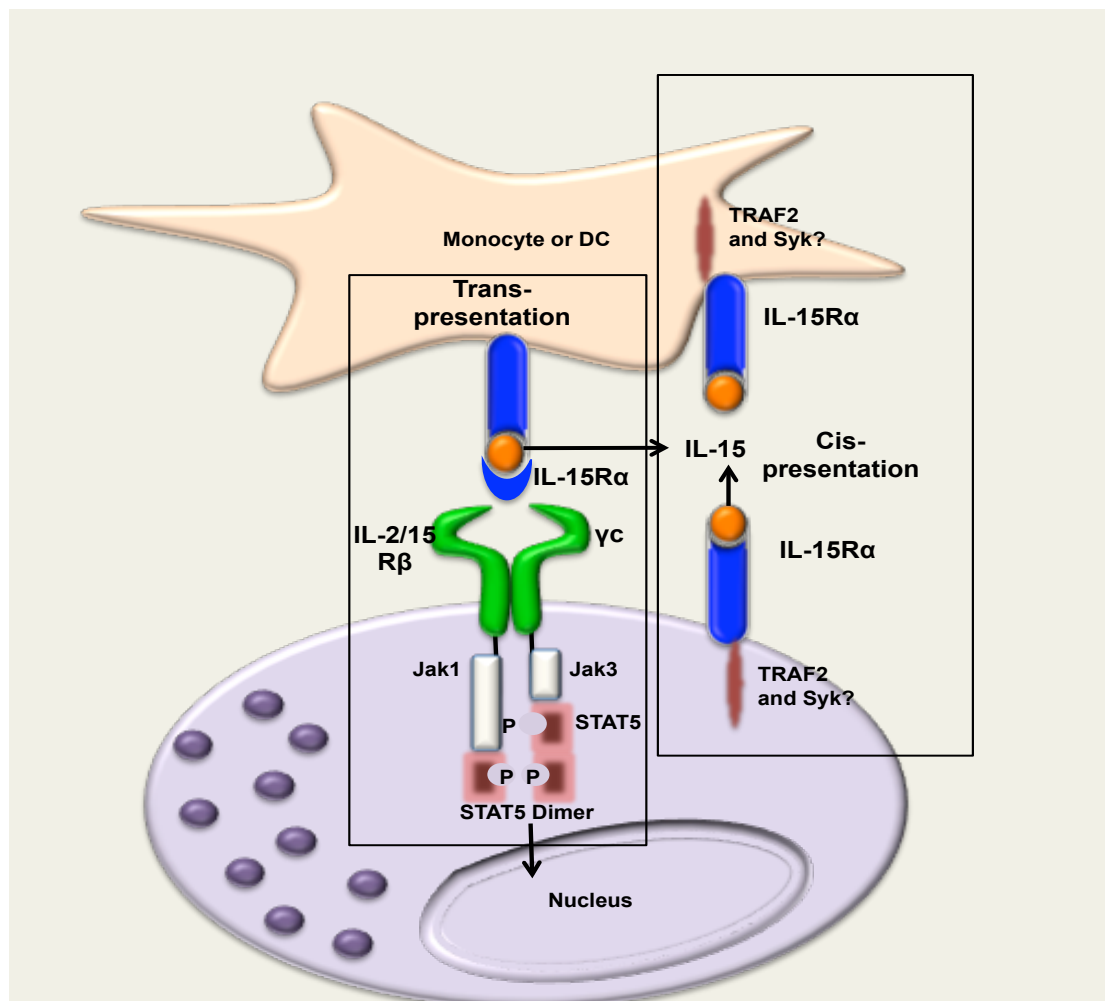


Figure 1.8 Mechanisms of IL-15 action via IL-15R α and IL-2/15R $\beta\gamma$ receptors. IL-15 presented in a membrane bound form via the IL-15R α subunit (*trans*-presentation) by APCs to IL-2/15R $\beta\gamma$ on NK cells. IL-15 can also be delivered as a soluble protein directly to NK cells (*cis*-presentation). “This figure has been reproduced from an open access article under creative commons attribution license” (Meazza et al., 2011).

1.3.5 Interleukin-18

IL-18 is a member of the IL-1 cytokine family that was initially known as an IFN- γ inducing factor (Dinarello, 1999). IL-18 was identified in mouse serum after the administration of endotoxins and was found to share similar properties with IL-12 (Okamura et al., 1995). IL-18 is closely related to IL-1 β , however, while IL-1 β expression is absent in healthy individuals, monocytes and epithelial cells of healthy individuals express IL-18 abundantly. Macrophages and DCs contain IL-18 precursors that are cleaved in a caspase-dependent manner and released outside the cell upon stimulation. In this context, it was shown that the interaction between immature DCs and NK cells leads to cytoskeleton rearrangement, increasing Ca²⁺ influx and followed by accumulation of secretory IL-18 at the DC/NK cell synapse (Semino et al., 2005). IL-18 induces the production of IFN- γ by T cells and NK cells (Nakanishi et al., 2001). Moreover, IL-18 induces upregulation of adhesion molecules and chemokine receptors by NK cells, and enhances cytotoxicity of NK cells (Mailliard et al., 2005).

The IL-18 receptor consists of two subunits, IL-18R α and IL-18R β (Fig 1.9). The alpha subunit plays key role in responses to IL-18, as IL-18R α -deficient mice do not display any response to IL-18 and are unable to initiate signalling via this pathway (Hoshino et al., 1999). The expression of IL-18 receptors is largely regulated by IL-12. In this context, IL-12 promotes the induction of IL-18R α , whereas IL-18 upregulates IL-12R β 2 thereby providing a positive loop of stimulation by the two cytokines. However, NK cells can be stimulated independently by each cytokine, in particular CD56^{bright} NK cells, as they constitutively express IL-12R β 2 and IL-18R α (Kunikata et al., 1998, Wang et al., 2000). Upon binding of IL-18 to its receptor, the NF- κ B signalling pathway is activated via the MyD88 and IRK signal transduction molecules (Takeuchi et al., 2000). The outcome of signalling is NF- κ B binding to the IFN- γ promoter within the nucleus to enhance transcription of IFN- γ (Kojima et al., 1999).

IL-18 participates in the development of T_H cell responses via the production of cytokines. Mailliard and colleagues showed that IL-18 stimulation also induces “helper” function of NK cells (Mailliard et al., 2005). Although IL-18 activated NK cells do not secrete IFN- γ spontaneously, induction of CCR7 expression allows them to migrate to LN. Thereby, exposure of NK cells to DC-derived cytokines (IL-12 and IFN-

α) as well as T cell-derived cytokines (IL-2) leads to production of high levels of IFN- γ by NK cells. Subsequently, NK cells are able to polarise the T_H response towards IFN- γ production by T_H1 cells (Agaugue et al., 2008, Mailliard et al., 2005). On the other hand, IL-18 has been also found to induce T_H2 responses by facilitating the production of IL-4 (Pollock et al., 2003).

IL-18 enhances NK cell cytotoxicity by upregulating the expression of FasL on NK cells (Hashimoto et al., 2003). Thus, IL-18 has been implicated as a mediator of tumour regression and as a potential immunotherapeutic cytokine. Intravenous administration of IL-18 to patients with solid tumours was well-tolerated with a significant response observed in a patient who demonstrated 69 % regression of tumour lesions (Robertson et al., 2006). However, a phase II clinical trial that aimed to treat metastatic melanoma concluded that IL-18 alone had limited anti-tumour activity (Tarhini et al., 2009). Currently, IL-18 is used to augment rituximab activity, in the treatment of non-Hodgkins lymphoma, by enhancing IFN- γ production and the ADCC pathway (Srivastava et al., 2013).

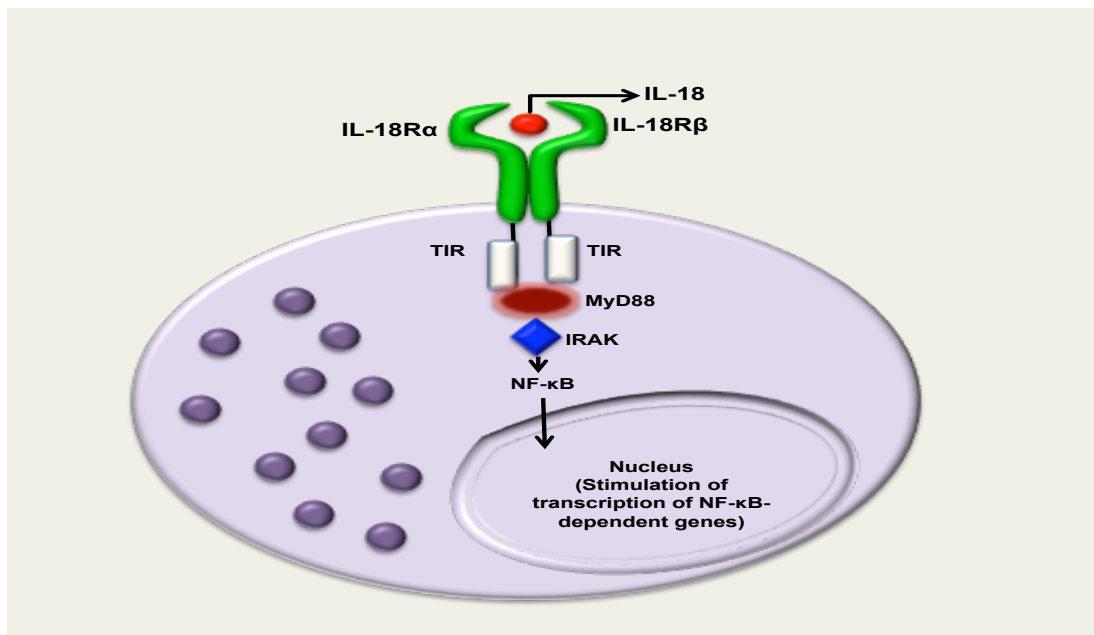


Figure 1.9 Activation cascade of the NK- κ B pathway triggered by IL-18. IL-18 produced by activated macrophages and DCs binds to IL-18 heterodimer receptor activating the components of the NK- κ B pathway. “This figure has been reproduced from an open access article under creative commons attribution license” (Alboni et al., 2010).

1.3.6 Interleukin-21

IL-21 is a γ c cytokine that is structurally similar to IL-2 and IL-15. The IL-21R is a homologue of the IL-2/IL-15R β subunit. IL-21 has both activating and inhibitory effects on lymphoid cells, as it enhances CTL function as well as inhibits antibody production by B cells. IL-21 also induces pleiotropic effects on NK cells. While IL-21 activation increases the size of NK cells and granule expression, it reduces their viability and proliferative capacity. Nonetheless, IL-21 potentially induces terminal maturation of NK cells associated with enhanced anti-tumour effects and cytokine secretion (Brady et al., 2004). The use of IL-21 for cancer immunotherapy is currently being assessed in a phase II clinical trial to treat non-Hodgkin's lymphoma, metastatic melanoma and renal cell carcinoma (clinicaltrials.gov, trial numbers; NCT00336986, NCT00389285 and NCT00347971) (Frederiksen et al., 2008).

1.4 Cancer immunotherapy

Immunotherapy strategies aim to enhance the recipients' immune response to help clear cancerous cells. Recipients may receive cytokine therapy to boost the function of their own NK cells and CTLs or receive direct cellular therapy. Cells that have the capacity to fight tumours have the potential to be expanded and adoptively transferred. In addition, in the context of haematological malignancies, patients may receive HSCT along with *ex vivo* expanded NK cells whereby both adoptively transferred and reconstituted NK cells mediate anti-tumour effects.

1.4.1 Haematopoietic stem cell transplantation

Early work in animal models revealed that irradiated recipients could be rescued by the transplantation of BM cells from syngeneic mice or with stored autologous cells. Thomas and colleagues pioneered haematopoietic stem cell transplantation (HSCT) by infusing BM from an identical twin to treat a patient who suffered from leukaemia (Thomas et al., 1959). After the identification of HLA in 1960, the transplantation of allogeneic HSC became more feasible. HLA genes are inherited as haplotypes from parents giving 1 in 4 probabilities of two siblings to be HLA-identical. In 1970, Thomas and colleagues treated patients suffering from end-stage leukaemia using BM of HLA-identical siblings establishing the foundation of HSCT (Thomas et al., 1977). Although HSCT is now mainly used to treat haematological and lymphoid cancers, it is also used

to treat other diseases including immunodeficiency, autoimmune disorders and anaemia. HSCs can be obtained from the host prior chemotherapy (autologous) or from a suitable HLA-matched donor (allogeneic). Treatment with autologous HSCs is safe, although autologous HSCT is not curative and is only used to prolong the life of patients. In contrast, the use of allogeneic HSC sources offers better outcomes in treating haematological malignancies although it is associated with complications (Copelan, 2006).

1.4.1.1 Preparative regimens

The objectives of preparative regimens are (i) to destroy the BM of the recipient prior transplantation and to suppress the recipient's immune system; (ii) to create space within the recipient BM for donor HSC engraftment; (iii) to destroy cancer cells and mediate an anti-tumour mechanism through presentation of tumorigenic antigens by APCs (Lake and Robinson, 2005). Several conditioning regimes are used to eradicate tumour cells and suppress the immune system. Total body irradiation (TBI) is a myeloablative approach that effectively eliminates cancer cells, however it is associated with toxicity and high transplantation related mortality. This led to the development of reduced intensity regimens, whereby the elimination of cancer cells depends primarily on the graft (Shimoni and Nagler, 2004). After transplantation, successful outcomes are associated with the ability of the graft to generate new lymphoid and myeloid cells, which is referred to as immune reconstitution. This is a very important determinant of HSCT outcome as slow immune reconstitution results in increased susceptibility to infections, especially in the myeloablative regime setting (Welniak et al., 2007).

1.4.1.2 Haematopoietic stem cell sources

BM is a rich source of HSC and was the only HSC source used until the 1990s. BM is collected by aspiration from iliac crests of donors under general anaesthesia and then infused into patients. One of the major complications after BM transplantation (BMT) is the high incidence of graft-*versus*-host disease (GvHD). Hence, T cell depletion (manipulated graft) is often performed, however, it may increase the risk of relapse and affect survival rates when compared to non-manipulated BM graft (Copelan, 2006, Lan et al., 2003).

As compared to BM, PB contains low numbers of HSC. However, HSCs can be collected in large quantities and harvested by apheresis following granulocyte-colony stimulating factor (G-CSF) infusion, a procedure which is also referred to as mobilisation (Bensinger et al., 2001, Schmitz et al., 1995). Mobilised PB (mPB) using G-CSF is currently a gold standard procedure because of the higher numbers of HSC obtained by apheresis, which promotes faster engraftment of neutrophils and earlier lymphocyte reconstitution. However, the incidence of chronic GvHD after mPB transplantation compared to BMT may be higher (Schmitz et al., 2005).

The first successful cord blood transplantation (CBT) was performed in a patient with Fanconi's anaemia in 1988 using cryopreserved CB from a healthy HLA matched sibling (Gluckman et al., 1989). Nowadays, CB is being increasingly used as an alternative source of HSC for transplantation. It has clinical advantages over other sources of HSC including off-the-shelf availability reducing waiting time for recipients. Indeed, more than 50 CB banks worldwide have been established with around 606,000 CB units available from registries (<http://www.bmdw.org/>). Furthermore, CBT allows for less stringent HLA-matching than other sources of HSC (Wagner et al., 1996) and importantly it shows reduced incidence and severity of GvHD whilst preserving the *graft-versus-leukaemia* (GvL) effect (Rocha et al., 2001). However, CB contains a lower number of HSC than BM or mobilised mPB resulting in slow immune reconstitution rendering the recipient susceptible to infections (Rocha et al., 2001, Laughlin et al., 2004, Gluckman, 2009). One of the main causes of CBT-related morbidity is CMV infection. CMV infection is reactivated in CMV seropositive CB recipients early after transplantation in whom no protective T cell responses have yet been developed (McGoldrick et al., 2013).

Early CBT procedures were performed only for children mainly because CB contains a low number of HSCs. Nevertheless, several strategies have been explored in order to increase the HSC dose in order to enable CBT to be performed in adult patients. These include infusing two CB units (Barker et al., 2005) or *ex vivo* expansion of CB HSC using mesenchymal stromal cells (de Lima et al., 2012), hence promoting faster engraftment. In addition, CB cells can be injected directly into the bone of the recipient to deliver better engraftment (Frassoni et al., 2008). In this context, it was shown in animal models that less than 20 % of HSC injected intravenously are able to reach the

BM (Cui et al., 1999). Hence, direct injection of CB into the BM prevents trapping of progenitors within tissues that do not support haematopoiesis and improves colonisation of HSC within the recipient BM (Brunstein and Wagner, 2006). In addition, another strategy to improve the outcome of CBT has been developed by co-infusion of CB alongside with purified HSCs from a haploidentical donor. This approach resulted in faster short-term and long-term engraftment and immune reconstitution (Fernández, 2009, Fernández et al., 2005)

Immune reconstitution varies among lymphocyte subsets after HSCT. In general, NK cells reconstitute first within a month after transplant. Within T cell subsets, the CD4⁺ T cell count reaches normal levels within 9 to 12 months, whereas reconstitution of CD8⁺ T cells begins from 4 months post-transplant and reaches normal values within 9 months. B cell count remains low, as it takes up to 12 months for B cells to recover post-transplantation (Petersen et al., 2003). After CBT, NK cell reconstitution in the host occurs as early as 2 months with detectable lytic function within one month. NK cells are therefore believed to play a key role in mediating GvL after CBT (Thomson et al., 2000, Béziat et al., 2009).

1.4.1.3 Early and delayed complications

Patients who undergo HSCT are susceptible to several complications due to immune deficiency as a result of the use of immunosuppressive drugs or due to delayed immune reconstitution. There are several complications that occur early after transplantation including mucositis (fungal infections) (Spielberger et al., 2004), sinusoidal obstruction syndrome (hepatomegaly, jaundice and fluid retention) (DeLeve et al., 2002), transplantation related lung disease (Cooke and Yanik, 2004), and viral infections including CMV (Boeckh et al., 2003) and EBV (Brunstein et al., 2006). Graft failure is another major complication that may occur after allogeneic transplantation. The incidence of graft failure increases in HLA-mismatched transplantation, unrelated transplantation or in patients treated with reduced intensity conditioning regimens. Graft failure corresponds to the absence of engraftment of donor cells after HSCT and is associated with graft rejection by the recipient immune system, mainly by recipient T cells (Mattsson et al., 2008).

Donor cells can attack recipient cells in a condition known as graft-*versus*-host disease (GvHD) (Ferrara et al., 2009). This disease is initiated when APCs prime donor T cells resulting in T cell activation and expansion and is associated with cytokine storm (Ferrara and Reddy, 2006). Donor activated T cells attack the skin, gut and liver of the recipient causing debilitating injuries. The incidence of GvHD, however, decreases the probability of tumour relapse since donor T cells also attack residual leukaemia cells remaining after chemotherapy (Apperley et al., 1986, Mackinnon et al., 1995, Kolb et al., 1995). GvHD can be treated with corticosteroids; yet, this could result in immunodeficiency and increased susceptibility to infections. Although GvHD occurs mainly as a result of HLA-mismatch, recipients of HLA-matched sources may still develop GvHD because of minor histocompatibility antigen (MiHA) mismatches between donor and recipient (Warren et al., 2012). Other risk factors including age and graft source may contribute to the increased incidence of chronic GvHD. In addition, the incidence of secondary cancers increases after transplantation, which could be a result of the type of chemotherapy used before transplantation (Curtis et al., 1997, Metayer et al., 2003).

1.4.1.4 Role of NK cells in haematological stem cell transplantation

NK cells have been shown to be key effectors of the GvL effect in HSCT (Ruggeri et al., 2002, Ruggeri et al., 2005). Ruggeri *et al.* reported the efficacy of alloreactive NK cells in inducing an anti-tumour effect in haematopoietic transplants. NK cells with KIR-ligand incompatibility (HLA-C1, C2 or Bw4) were able to lyse recipients' APC and hence reduced the incidence of GvHD, in addition to leukaemia cell lysis providing GvL. Acute myeloid leukaemia-engrafted NOD-*scid* mice infused with alloreactive NK cells have been shown to be able to clear leukaemia cells, indicating their role in the GvL effect (Ruggeri et al., 2002, Ruggeri et al., 2005). However, the beneficial effect of KIR-ligand incompatibility in mediating NK cell alloreactivity has remained controversial, as several groups have not observed any influence of KIR mismatch. This may be due to differences in transplantation procedure, graft source, underlying malignancy or T cell manipulation (T cell depletion or repletion) (Davies et al., 2002, Farag et al., 2006, Giebel et al., 2003, Kroger et al., 2006, Lowe et al., 2003).

1.4.2 Tumour immune evasion

Cancerous cells have evolved mechanisms to evade the effector functions of immune cells. Tumour cells can produce immunosuppressive cytokines such as TGF- β or IL-10 to suppress effector mechanisms, and thereby establish an immunoprivileged microenvironment (Aruga et al., 1997, Mamessier et al., 2011). Tumour cells often skew the adaptive response towards T_H2 immunity which is less effective in combating tumours (Aruga et al., 1997). Moreover, tumour cells suppress DC maturation by altering the expression of IL-6, IL-10 and GM-CSF (Morse et al., 2002). In some malignancies, regulatory T cells expand and suppress the specific function of T_H1 cells and CTL (Yamaguchi and Sakaguchi, 2006). Furthermore, tumour cells downregulate or shed NKG2D ligands such as MICA/MICB or UL-16 binding protein and evade NK cell effector functions (Salih et al., 2002, Waldhauer and Steinle, 2006). Furthermore, malignant cells in certain conditions eliminate effector immune cells by AICD or via FasL (Saff et al., 2004).

1.4.3 NK cell-based therapy

Various strategies have been developed to overcome an inadequate anti-tumour immune response. In this context, NK cells provide a potential cellular source for cancer immunotherapy. NK cells induce direct killing of tumour cells and assist with the differentiation of antigen-specific anti-tumour responses (Fig 1.10). The following section discusses strategies that have been implemented to exploit NK cell effector function as an immunotherapeutic source (Table 1.5).

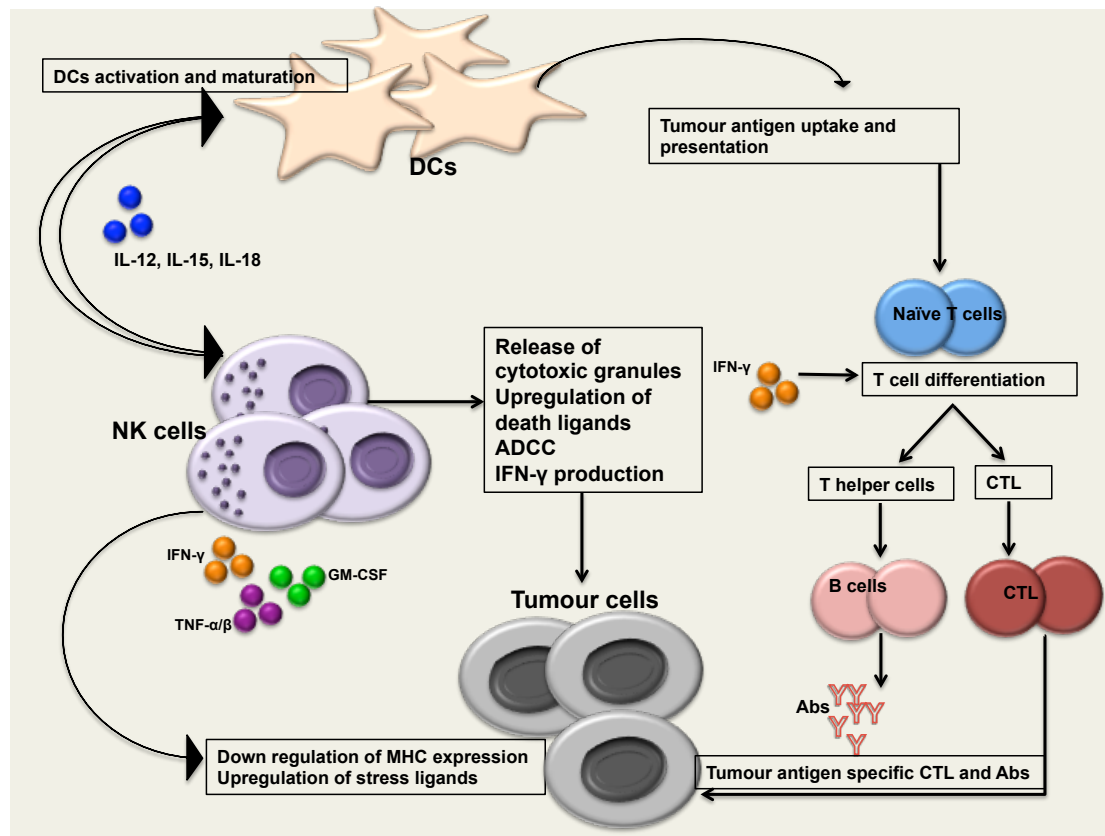


Figure 1.10 NK cell anti-tumour responses. NK cell effector functions are triggered by cells that have downregulated MHC class I expression or upregulated stress ligands. Tumour cells are killed directly by exocytosis of cytotoxic granules, death receptor-pathway activation or ADCC. Cytokines released by DCs trigger the production of inflammatory cytokines such as IFN- γ and TNF- α by NK cells that, in turn, enhance further activation and maturation of DCs. Mature DCs uptake and present tumour-derived antigens to naïve T cells that leads to T cell differentiation into effector T_H cells and CTLs. The production of IFN- γ by NK cells also primes T cells directly. While CTLs mediate direct elimination of tumour cells, interactions between T helper cells and B cells results in the production of tumour antigen specific-antibodies (Cheng et al., 2013). License obtained from Nature Publishing Group; 3397701357939.

Table 1.5 NK cell-based therapy strategies

NK cell-based therapy	Effect(s)	Limitation(s)
Stimulation of autologous NK cells with cytokines (IL-2, IL-12, IL-15, IL-18 and IL-21), adoptive transfer of <i>ex vivo</i> stimulated NK cells with or without systemic administration of cytokines	Upregulation of activating receptors and adhesion molecules and enhanced NK cell functionality associated with upregulation of perforin and granzymes.	Toxicity of cytokine administration, suppression of NK cell activity by self-MHC molecules
Stimulation of autologous NK cells with antibodies (IFN type I, KIR)	NK cell limited anti-tumour activity against many types of tumours	
Adoptive transfer of un-stimulated donor NK cells	NK cell alloreactivity mediated by KIR mismatch	Cells cannot be maintained in culture and difficult to access the same donor for several infusions
Adoptive transfer of <i>ex vivo</i> cytokine expanded allogeneic NK cells (IL-15)	Enhanced cytotoxicity, large-scale expansion of NK cells to allow multiple infusions	Exhaustion and cytokine addiction
Systemic administration of tumour specific monoclonal antibodies stimulating the ADCC pathway	Efficient killing of Ab-coated cells by ADCC Many drugs are in use; Rituxumab to treat non-Hodgkin's lymphoma (NHL) patients and Trastuzumab/Herceptin to treat metastatic breast and gastric carcinoma patients	Drug toxicity
Adoptive transfer of <i>ex vivo</i> expanded NK cell lines	Enhanced killing capacity and cytokine production by NK cells Large-scale production of NK cells	Rejection by recipient's immune system
Adoptive transfer of genetically modified NK cells (upregulation of activating receptors or suppression of inhibitory receptors by interference with transcription genes)	Effective killing of tumour cells and enhanced signalling via activating receptors	Limited specificity of NK cell anti-tumour activity

1.4.3.1 Autologous NK cells

Initial studies proposed that NK cell anti-tumour activity could be enhanced by systemic administration of cytokines. Administration of IL-2 (Burns et al., 2003, Rosenberg et al., 1987), IL-12 (Atkins et al., 1997), IL-18 (Nakamura et al., 2000), IL-21 (Frederiksen et al., 2008) and type I IFN (Kantarjian et al., 1995) stimulated the production of host LAK cells. Cells displayed enhanced cytotoxicity, upregulated adhesion molecules and activating receptors, increased expression of perforin, FasL and TRAIL, and produced increased amounts of inflammatory cytokines. Despite the greater NK cell cytotoxicity, these studies showed limited success and life-threatening toxicities (Smyth et al., 2004). A phase I clinical trial that tested the safety and feasibility of using a Heat shock protein-70 peptide and IL-2 *ex vivo* activated autologous NK cells to treat colon and lung cancer showed enhanced cytotoxic function of NK cells (Krause et al., 2004). However, in IL-2 based trials, expansion of Tregs was observed that resulted in the induction of AICD and inhibition of NK cell effector function against malignant cells. Accordingly, this strategy was optimised by using adoptive transfer of *ex vivo* IL-2 activated NK cells showing better outcomes than the systemic administration of IL-2 (Barkholt et al., 2009). Recently, it was shown by Miller and colleagues that depletion of Tregs using IL-2 diphtheria toxin before the infusion of *ex vivo* IL-2 activated NK cells enhances their expansion in AML patients by up to 1000 NK cells/ μ L (Bachanova et al., 2014). Similarly, transfer of *ex vivo* expanded autologous NK cells with IL-15 and hydrocortisone resulted in effective anti-tumour responses against lung cancer cells in a mouse model. In this model, high doses of IL-15 were required for effective elimination of cancer cells where IL-15 was trans-presented in a membrane-bound form via the IL-15R α leading to enhanced tumour immunosurveillance *in vivo* (Kobayashi et al., 2005).

Although the use of autologous NK cells together with the systemic administration of cytokines shows considerable success, this strategy has several limitations. First, the administration of cytokines results in toxicity and cytokine-induced NK cell apoptosis. Second, the cytotoxicity of NK cells against tumour cells observed *in vitro* was not reflected *in vivo*. In this context, the effector function of autologous NK cells is inhibited by interaction with self-MHC molecules on leukaemia targets (Miller et al.,

2005). Accordingly, allogeneic NK cell-based therapies may provide a better option than autologous NK cells in terms of providing an anti-tumour effect.

1.4.3.2 Allogeneic NK cells

Allogeneic NK cells offer greater cytotoxicity than autologous NK cells and have been shown to be effective at controlling AML relapse (Miller et al., 2007, Miller et al., 2005, Ruggeri et al., 2002). Allogeneic NK cells induce killing of leukaemia blast when MHC class I alleles on target cells are incompatible with KIR epitopes expressed by NK cells (Ruggeri et al., 2002, Ruggeri et al., 2005). Currently, criteria for selection of haploidentical donors with KIR type mismatches against the recipient's MHC class I have been established (Pende et al., 2009). In addition, allogeneic NK cells have been shown to be potent against solid tumours. This strategy has proven to be safe and effective to treat several types of cancers including metastatic melanoma, renal cell carcinoma, Hodgkin's disease and poor-prognosis AML (Miller et al., 2005). Furthermore, allogeneic NK cells can be expanded *in vitro*. Allogeneic NK cells expanded with IL-15 and hydrocortisone are being tested for the treatment of non-small cell lung carcinoma in a phase I safety clinical trial. Expanded allogeneic NK cells have been shown to be safe and effective for the treatment of adenocarcinoma and squamous cell carcinoma (Iliopoulou et al., 2010). Thus, the use of IL-15 with allogeneic NK cells could provide a good basis for future immunotherapy. Unlike IL-2, IL-15 activated NK cells survive the methylprednisolone treatment promoting potential use of this cytokine in patients treated with steroids (Chiossone et al., 2007).

1.4.3.3 Antibody-based NK cell therapy

CD16 expression by NK cells enables them to bind to antibody-coated targets initiating the ADCC pathway that eventually results in elimination of target cells. Antibody-based drugs are widely used in cancer therapy. In particular anti-CD20 (Rituxumab) is used to treat non-Hodgkin's lymphoma (Maloney et al., 1997), anti-HER2 (Trastuzumab) is used to treat metastatic breast cancer and gastric carcinoma (Hofmann et al., 2008, Pegram et al., 1998) and anti-CD52 (Alemtuzumab) is used to treat B-CLL and GvHD post-HSCT (Ratzinger et al., 2003). Antibody-based therapies have been modified to reduce toxicity by humanisation of antibodies and alleviation of complement activation (Alderson and Sondel, 2011). In addition, the anti-tumour effect

mediated via the ADCC pathway could be enhanced by co-administration of cytokines, TLR agonists or using antibodies that stimulated NK cell activating receptors or block NK cell inhibitory receptors (Alderson and Sondel, 2011, Iannello and Ahmad, 2005).

In MHC class I expressing tumour cells, effector functions of autologous NK cells are often inhibited by KIR. Thus, strategies to block KIR expression using antibodies have been developed to potentiate NK cell cytotoxicity (Romagné et al., 2009). The use of anti-KIR (IPH2101) is currently being tested in a phase I clinical trial to treat relapsed/refractory multiple myeloma (clinicaltrials.gov, trial number; NCT00552396) (Benson et al., 2012).

1.4.3.4 NK cell lines

There are several immortalised NK cell lines that are able to mediate anti-tumour activity. These include NK-92, KHYG-1, NKL and NKG. Although the NK-92 cell-line lacks expression of KIR, it is able to induce target killing by exocytosis and is the only cell line that has received FDA approval. NK-92 has been tested as a therapy for renal cell carcinoma and advanced malignant melanoma and has been demonstrated to be beneficial and safe (Tonn et al., 2001). KHYG-1 and NKL showed greater anti-tumour activity than NK-92, however, administration of these cell lines into human remains to be tested (Cheng et al., 2011, Suck et al., 2005). The main advantage of using NK cell lines is the ability to maintain their expansion *in vitro* under good manufacturing practice (GMP) conditions, thereby yielding large-scale NK cell products for NK cell-based immunotherapy.

1.4.3.5 Genetic manipulation of NK cells

Genetic manipulation of NK cells could be an effective approach to mediate specific NK cell anti-tumour effects against different targets. This approach involves genetic transfer of chimeric receptors specific for an antigen or manipulating the expression of activating and inhibitory receptors. For example, the transduction of a CD19 chimeric receptor into NK cells enhanced NK cell killing capacity against malignant B cells (Imai et al., 2005). Similarly, the genetic modification of the NK-92 cell line using a chimeric receptor that contains a CD20-specific fragment increases NK cell cytotoxicity against CD20⁺ targets (Müller et al., 2008). This approach has several

limitations including the use of murine derived chimeric receptor that could trigger the host immune response, the persistence of genetically modified NK cells after adoptive transfer and limited specificity of the anti-tumour activity by NK cells being mediated by the transduced receptors.

1.4.3.6 NK cell differentiation from haematopoietic stem cells

Over the two past decades, models aiming to investigate NK cell differentiation from HSCs have been established. The main purpose of these models was to study NK cell ontogenesis. However, the establishment of these models has also proven to be useful to generate high numbers of NK cells *in vitro*. HSCs can be obtained from different sources including BM (Miller et al., 1994, Mrozek et al., 1996), mPB (Yoon et al., 2010), human embryonic HESCs (Woll et al., 2009) or CB (Luevano et al., 2012b, Spanholtz et al., 2010). In these protocols, the BM microenvironment is provided by the addition of cytokines such as IL-7, IL-15, SCF and Flt3L, which are involved in commitment of HSCs to the lymphoid lineage and differentiation of progenitors into NK cell precursors. This approach is promising, as large number of NK cells can be generated from fresh or frozen HSCs after only a few weeks of culture (Luevano et al., 2014). However, the use of *in vitro* generated NK cells for clinical application is associated with several obstacles. Most protocols use animal-derived sera and cytokines in addition to feeder cell lines that currently makes them unsuitable for clinical use.

CB CD34⁺ HSCs appear to be the most promising HSC source for large-scale production of NK cells. Different groups have described distinct protocols to generate NK cells from CB CD34⁺ HSCs. Perez and colleagues generated NK cells with high expression of NKG2D, perforin, NKp46 and high cytotoxicity (Perez et al., 2006). Similarly, Grzywacz *et al.* produced NK cells with high expression of NKp44 and NKp30 that exhibited a killing capacity of almost 50 % of K562 at an effector to target ratio of 5 to 1 (Grzywacz et al., 2006). Other groups successfully generated NK cells from CB CD34⁺ HSCs with varied purity, phenotype and cytotoxicity which could have resulted from using different feeder layers and cytokines in different protocols (Bonanno et al., 2009, Frias et al., 2008, Haddad et al., 2004, Luevano et al., 2014). Spanholtz *et al.* established a GMP cytokine-based culture-system for the expansion of NK cells from fresh or frozen CB CD34⁺ HSCs using clinical-grade reagents

(Spanholtz et al., 2010). With purity exceeding 95 %, 3-log NK cell expansion from frozen CB CD34⁺ HSCs and 4-log NK cell expansion from fresh CB CD34⁺ HSCs could be achieved. NK cells generated using this approach express high levels of NKG2D and NCRs and are able to kill melanoma and myeloid cell lines, as well as primary AML cells *in vitro*. The generated NK cells are CD56⁺CD16⁺ with up to 60 % being NKG2A⁺ and 10 % KIR⁺, with only a minor population being NKG2A⁻KIR⁻. As NK cells were generated in 24-well plates, the same group has modified the protocol into a fully closed two-step culture system for clinical use (Spanholtz et al., 2011). Firstly, cryopreserved CB units were thawed and then CD34⁺ HSCs were enriched using a closed CliniMACS system. Secondly, automated bioreactors (gas-permeable culture bags) were used to generate NK cells in a feeder-free system as shown previously by Sutlu *et al.* (Sutlu et al., 2010). The purity of the final product was 90 % with a 2000-fold expansion of NK cells and the generated NK cells express NCRs and showed enhanced cytotoxic activity against K562 cells (Spanholtz et al., 2011). In addition, Shah and colleagues developed a protocol for large-scale expansion of CB NK cells using K562-derived artificial APCs (aAPCs) as a feeder layer (Shah et al., 2013). The use of aAPC-expanded CB NK cells following autologous HSCT to treat multiple myeloma is currently being tested in a phase I clinical trial (clinicaltrials.gov, trial number; NCT01729091). Another trial is underway to treat AML using *ex vivo* expanded NK cells from non-HLA matched CB CD34⁺ progenitors (clinicaltrials.gov, trial number; NCT01031368).

1.5 Aims of the study

NK cells mediate a potent anti-tumour effect providing an immunotherapeutic option to treat cancers. NK cells can be readily obtained from PB or CB. The latter source is a potentially valuable source of NK cells, as CB NK cells constitute up to 30 % of the total CB mononuclear cell population (Kotylo et al., 1990). In addition, CB NK cells have been shown to mediate GvL in CBT settings (Béziat et al., 2009). However, CB NK cells have been previously shown to exhibit poor cytotoxicity when resting, and to have an immature phenotype (Gaddy et al., 1995, Luevano et al., 2012a, Wang et al., 2007). Activation with cytokine can enhance the cytotoxic potential of CB NK cells (Dalle et al., 2005, Gaddy et al., 1995, Luevano et al., 2012a). However, previous studies have shown that CB NK cells require significantly higher doses of IL-2 for activation compared to PB NK cells (Condiotti et al., 2001, Luevano et al., 2012a), which could be suggestive of differential mechanisms of activation for PB and CB NK cells. Therefore, I hypothesise that the different activation mechanisms for PB and CB NK cells may imply distinctive immunological roles of NK cells in neonates and in adults. So far, studies that have investigated CB NK cell activation are incomplete. In this context, it was shown that CB NK cells become cytotoxic after stimulation with IL-2 (Luevano et al., 2012a, Xing et al., 2010), IL-12 (Gaddy et al., 1995), IL-15 (Dalle et al., 2005) or IL-18 (Nomura et al., 2001). However, collective analysis of the effect of each cytokine on CB NK cells when compared to PB NK cells has not yet been performed. Furthermore, although a few studies have assessed intracellular production of inflammatory cytokines including IFN- γ and TNF- α by NK cells (Dalle et al., 2005, Krampera et al., 2000, Luevano et al., 2012a, Wang et al., 2007), no study has assessed cytokine secretion by CB NK cells following cytokine stimulation. Furthermore, the migratory capacity of activated CB NK cells has not been assessed. The finding that CB NK cells require higher doses of IL-2 for activation and proliferation (Condiotti et al., 2001, Luevano et al., 2012a) is suggestive that IL-2 might not be the ideal cytokine to induce fully functional CB NK cells. Besides IL-2, there are many cytokines that induce biological effects on NK cells including IL-15, which is critical for NK cell development, survival, effector functions and proliferation; IL-12 and IL-18, which both induce NK cell cytotoxicity and cytokine production. Hence, the main aim of this study is to perform a comprehensive analysis of CB NK cell activation following

stimulation with IL-2, IL-12, IL-15, IL-18, the combination of IL-15+IL-2 and IL-15+IL-18 to compare with PB NK cells as follows:

1. Comparative analysis of the phenotypic characteristics of cytokine-activated PB and CB NK cells: this analysis focuses on the expression of activating and inhibitory receptors as well as the study of cytokine receptors and their signalling components (Chapter 3).
2. Functional analysis of cytokine-activated PB and CB NK cells: this study aims to measure NK cell effector functions including NK cell proliferation, cytotoxicity and inflammatory cytokine production following stimulation with different cytokines (Chapter 4).
3. Analysis of the NK cell trafficking repertoire: this analysis focuses on the upregulation of LN homing receptors by PB and CB NK cells as well as assessment of NK cell migration *in vitro* following cytokine stimulation (Chapter 5).
4. The production of memory-like NK cells using cytokines: this study aimed to generate memory-like NK cells from PB and for the first time from CB using cytokines (Chapter 6).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents

All the reagents used in this thesis are listed in Table 2.1.

Table 2.1 List of reagents

Reagent	Company
10X Phosphate buffered saline (PBS)	Lonza, Belgium
1450 microbeta Plus liquid Scintillation	Perkin Elmer, UK
Absolute Ethanol	VWR BDH Prolabo, UK
BD Pharm Lyse	BD Biosciences, UK
Carboxyfluorescein succinimidyl ester (CFSE)	Life Technologies, UK
⁵¹ Chromium	Perkin Elmer, UK
Distilled water	Baxter, Switzerland
Ethylenediaminetetraacetic acid (EDTA) 0.5 M ultra pure pH 8.0	Life Technologies, UK
Ficoll—Paque PLUS	GE Healthcare, Sweden
Heparin sodium 1000 IU/mL	Sigma, UK
Lympholyte-H	VH Bio Ltd., UK
Penicillin and streptomycin mixture (Pen-Strep)	Lonza, Belgium
RPMI 1640	Lonza, Belgium
Trisodium citrate	Sigma-Aldrich, USA
Triton 100X	VWR International, USA
Trypan blue (0.4 %)	Sigma, UK
Turk's stain	Sigma, UK
β-Mercaptoethanol (β-ME)	Life Technologies, UK

2.1.2 Materials

All materials used in this thesis are listed in Table 2.2.

Table 2.2 List of materials

Materials	Company
Falcon tubes (15 and 50 mL)	Sarstedt, Germany
HTS transwell 96-well plate with 5- μ m polycarbonate membrane	Corning Life Technologies, USA
LS columns	Miltenyi Biotech, Germany
Minisart sterile filter (0.45 μ m)	Sartorius Stedim Biotech, Germany
Multi-stand MACS Magnet	Miltenyi Biotech, Germany
Partec CellTrics 30 micron filters	Partec, Germany
Pasteur pipettes	Fisher, UK
Serological pipettes (5, 10 and 25 mL)	Sarstedt, Germany
Tissue culture flask (25, 75 and 150 cm ²)	Sarstedt, Germany
U-bottom 96-well plates	Sarstedt, Germany
V-Bottom 96-well plates	Sarstedt, Germany

2.1.3 Serum

The sera used for cell culture and analysis are listed in Table 2.4.

Table 2.3 List of sera

Serum	Manufacturer
Bovine Serum Albumin (BSA) fraction V	Calbiochem, Germany
Bovine Serum Albumin	Sigma, UK
Foetal bovine serum (FBS)	Lonza, Belgium
Human serum type AB (AB serum)	Lonza, Belgium

2.1.4 Buffers

All buffers used for cell staining, isolation and washing are listed in Table 2.3.

Table 2.4 List of buffers

Buffer	Components
FACS buffer	1X PBS and 10 % v/v FBS
Lysing buffer	10X BD Pharm Lyse diluted to 1X with distilled water
MACS buffer	1X PBS, 1 % w/v BSA and 2 mM EDTA
PBS	10X PBS diluted to 1X with distilled water

2.1.5 Media

All the media used for cellular studies are listed in Table 2.5.

Table 2.5 List of media

Medium	Components
Culture medium	RPMI 1640, 5 μ M β -ME, 1 % Pen-Strep and 10 % v/v FBS
Cytokine-induced memory NK cell medium	RPMI 1640, 5 μ M β -ME, 1 % Pen-Strep and 10 % v/v AB Serum
Transport medium	RPMI 1640, 33 % w/v Tri-sodium citrate, 5 μ M β -ME
Transwell migration medium	RPMI 1640, 5 μ M β -ME, 1 % Pen-Strep and 0.5 % w/v BSA fraction V

2.1.6 Kits

All the commercial kits used for cellular isolation, RNA extraction, signalling pathway analysis and cytokine analysis are listed in Table 2.6.

Table 2.6 List of kits

Kit	Manufacturer
Cytofix/Cytoperm TM plus	BD Biosciences, USA
Human Granulocyte Depletion Kit	Stem Cell Technologies, France
Human T _H 1/T _H 2 11plex Ready-to-Use Kit FlowCytomix	eBioscience, Austria
NK Cell Isolation Kit	Miltenyi Biotec, Germany
Phosflow Starter Kit	BD Bioscience, USA
RNeasy Mini kit	Qiagen, Germany

2.1.7 Cytokines

All the cytokines described in this thesis are listed in Table 2.7.

Table 2.7 List of cytokines

Cytokine	Provider
IL-2	ProSpec, Israel
IL-12	PeproTech, UK
IL-15	PeproTech, UK
IL-18	MBL International Co. Ltd, USA

2.1.8 Monoclonal antibodies

The antibodies used for flow cytometry analysis are listed in Table 2.8.

Table 2.8 List of antibodies

Marker	FC*	Clone	Isotype	Manufacturer
7AAD	-	-	-	BD Pharmingen
Annexin V	FITC	-	-	BD Pharmingen
CCR7	PE	3D12	Mouse IgG2a k	BD Pharmingen
CCR7	PE	150503	Mouse IgG2a k	R&D Systems
CD3	PerCP	SK7	Mouse IgG1 k	BD Pharmingen
CD3	PE	SK7	Mouse IgG1 k	BD Pharmingen
CD16	FITC	NKP15	Mouse IgG1 k	BD Pharmingen
CD16	APC	3G8	Mouse IgG1 k	Biolegend
CD56	PE-Cy5	B159	Mouse IgG1 k	BD Pharmingen
CD56	APC	B159	Mouse IgG1 k	BD Pharmingen
CD62L	FITC	Dreg56	Mouse IgG1 k	BD Pharmingen
CD69	APC	L78	Mouse IgG1 k	BD Pharmingen
CD94	FITC	HP-3D9	Mouse IgG1 k	BD Pharmingen
CD158a	FITC	HP-3EA	Mouse IgM k	BD Pharmingen
CD158b	FITC	CH-L	Mouse IgG2b k	BD Pharmingen
CXCR3	APC	1C6/CXCR3	Mouse IgG1 k	BD Pharmingen
IFN- γ	FITC	4S.B3	Mouse IgG1 k	BD Pharmingen
CD25 (IL-2R α)	APC	BC96	Mouse IgG1 k	eBioscience
CD122 (IL-2R β)	PE	Mik-B3	Mouse IgG1 k	Pharmingen
CD212 (IL-12R β 1)	APC	2.4 E6	Mouse IgG1 k	Pharmingen
CD212 (IL-12R β 2)	PE	2B6/12B2	Rat IgG2a k	Pharmingen

IL-15R α	PE	eBioJM7A4	Mouse IgG2b	eBioscience
IL-18R α	FITC	H44	Mouse IgG1 k	eBioscience
NKG2A	PE	Z199	Mouse IgG2b k	Beckman-Coulter
NKp44	APC	P44-8	Mouse IgG1 k	Biolegend
p-Stat4	PE	pY693	Mouse IgG2b	BD Pharmingen
p-Stat5	PE	pY694	Mouse IgG1 k	BD Pharmingen

FC*= Fluorochrome

2.1.9 Molecular biology techniques

All the reagents and primers used to perform molecular biology techniques are listed in Tables 2.9 and 2.10.

Table 2.9 List of reagents

Reagent	Company
0.1 M DTT (0.05 mM/mL)	Life Technologies, UK
2X qPCR MasterMix	Primer Design, UK
5X First-Strand Buffer	Life Technologies, UK
dNTPs (0.1 μ mol/ μ L)	Bioline, UK
Optical reaction 96-well plates with Barcode	Applied Biosystems, US
Random primers (0.5 μ g/ μ L)	Promega, UK
Recombinant RNAsin Ribonuclease Inhibitor (40 u/ μ L)	Promega, UK
SuperScript III RT 200 U/ μ L	Life Technologies, UK
Water for injections BP	B. Braun, Germany

Table 2.10 List of primers

Gene	Forward primer (F) and Reverse primer (R)	Conc.	Reference
BCL-2	F: 5'-CTGCACCTGACGCCCTTCACC-3' R: 5'-CACATGACCCACCGAACTCAAAGA-3'	600 nM	(Ge et al., 2006)
Granzyme B	F: 5'-TGGGGGACCCAGAGATTAAAA-3' R: 5'-TTTCGTCCATAGGAGACAATGC-3'	300 nM	(Morissette et al., 2007)
IFN- γ	F: 5'-TGG CTC TGC ATT ATT TTT C-3' R: 5'-CGC TCC CCA AGA AGA C-3'	300 nM	(Gober et al., 2007)
Perforin	F: 5'-CGCCTACCTCAGGCTTATCTC-3' R: 5'-CCTCGACAGTCAGGCAGTC-3'	900 nM	(Morissette et al., 2007)

2.1.10 Instrumentations and software

Instrumentation and software used for molecular biology work are provided in table 2.11.

Table 2.11 Instrumentations and software

Instrument	Software
7500 Real Time PCR System (AB Applied Biosystems)	Sequence detection software version 4.1
FACSCalibur TM Flow Cytometer (BD, US)	FlowJO version 6.4.2 FlowCytomixPro version 3.0
NanoDrop-1000 Spectrophotometer (US)	NanoDrop-1000 version 3.3.1
Perkin Elmer 1450 liquid scintillation counter	Wallac microbeta windows work station Version 3.20.002

2.2 Blood Samples

2.2.1 Cord blood

All CB units were obtained from the Anthony Nolan Cord Blood Bank in Nottingham (NREC 10/H0405/27), UK, under written informed consent from normal or caesarean deliveries. CB units were collected into bags containing citrate-phosphate-dextrose anticoagulant. All samples were processed within 24 h upon reception, and 24 to 48 h after collection.

2.2.2 Peripheral blood

Healthy adult volunteers from Anthony Nolan donated PB with written informed consent. Samples were anticoagulated with heparin (1 IU/mL).

2.3 Methods

2.3.1 Mononuclear cell isolation

Mononuclear cells from CB and PB were isolated using a density gradient separation method. CB samples were incubated prior to mononuclear cell isolation with 5 $\mu\text{L/mL}$ of RosetteSep® Human Granulocyte depletion kit to deplete granulocytes. Samples were diluted prior to separation by adding an equal volume of blood to transport media (RPMI 1640 media supplemented with 33 % w/v trisodium citrate and 5 μM of $\beta\text{-ME}$). The diluted blood was then layered on Lympholyte-H for PB or on Ficoll-Paque PLUS for CB at a 2:1 ratio and then centrifuged at 850 x g for 30 min without break to avoid disturbance of the cellular interface. The mononuclear cell layer was carefully aspirated with a Pasteur pipette and residual red blood cells were lysed using 1X BD PharmaLyse for 3 min at 37 °C.

2.3.2 Cellular enumeration

Cell counts were performed using a haemocytometer and aDMLB microscope (Leica, Germany). Trypan blue was used to determine cellular viability and Turk's staining was used to determine the total number of nucleated cells.

2.3.3 NK cell isolation

NK cell isolation was carried out by negative selection using the NK Cell Isolation Kit II (Miltenyi Biotec) following manufacturer's recommendations with washing modifications. Mononuclear cells from PB or CB were incubated with biotin-conjugated antibodies and then with the NK cell MicroBead cocktail at 4 °C. Erythrocytes, granulocytes, monocytes, T lymphocytes, B lymphocytes and stem cells were directly labelled with the antibody cocktail, whereas NK cells were left untouched. Cell suspensions were then applied to the columns passing through Partec CellTrics filters. Columns were washed twice with 7 mL of MACS buffer (Table 2.2) and only NK cells were isolated. NK cells were spun down for 10 min at 300 x g and resuspended in RPMI containing 10 % v/v FBS. The purity of the isolated NK cells was determined by flow cytometry as CD3⁻ CD56⁺ cells (Fig 2.1). The mean percentage of

purified NK cells from CB was $92.27 \% \pm 4.5$ and $90 \% \pm 2.2$ from PB. This data represents the purity of all PB and CB NK cells included in this thesis and are shown as mean \pm SD.

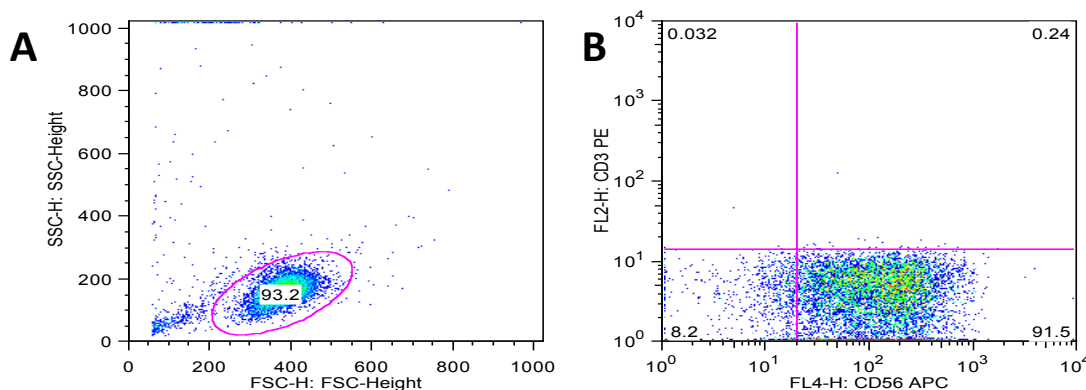


Figure 2.1 Assessment of NK cell purity. The purity of isolated NK cells (CB) was confirmed by flow cytometry. A) Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC). B) Isolated NK cells are CD3⁻CD56⁺.

2.3.4 NK cell culture

PB or CB NK cells were cultured with IL-2, IL-12, IL-15, IL-18, or the combination of IL-15 and IL-2 or IL-15 and IL-18. Various concentrations of these cytokines have been used by others to activate CB NK cells (Condiotti et al., 2001, Gaddy et al., 1995, Nomura et al., 2001, Satwani et al., 2011). Therefore, I tested higher and lower doses of IL-12 and IL-15 as it was previously observed that CB NK cells require up to 5 times more IL-2 (Condiotti et al., 2001, Luevano et al., 2012a). For IL-18, I followed the protocol of Nomura and colleagues where they showed optimal activation of CB NK cells using 100 ng/mL IL-18 compared to other doses (Nomura et al., 2001). NK cells were activated with IL-12 and IL-18 for 40 h as these cytokines induce apoptosis upon prolonged stimulation (Huang et al., 2010, Huang et al., 2011), whereas NK cells were cultured with IL-2 and IL-15 for 5 days due to the ability of these cytokines to promote NK cell proliferation and expansion (Becknell and Caligiuri, 2005, Carson et al., 1994).

Culture medium was prepared by adding 1 % Pen-Strep, 5 μ M β -ME and 10 % v/v FBS to RPMI 1640. Lyophilised human recombinant cytokines were reconstituted in PBS supplemented with 0.1 % w/v BSA or according to manufacturer's recommendations. Cytokines were diluted in culture medium prior to use. A volume of 1 mL of media

containing cytokines was added to 1×10^6 NK cells (refer to Table 2.12 for cytokine concentrations used). Cells were cultured in 96 well-round bottom plates at 37 °C, 5 % CO₂. For cultures longer than 40 h, the medium was changed by hemi-depletion (splitting cellular pellet into two wells followed by the addition of fresh culture medium).

Table 2.12 Cytokine concentrations and incubation times used.

Cytokine	PB NK cells	CB NK cells	Incubation time
IL-2	200 IU/mL (Chiossone et al., 2007)	1000 IU/mL (Condiotti et al., 2001)	5 days
IL-12	10 ng/mL (Cooper et al., 2001c)	10 or 50 ng/mL	40 h
IL-15	20 ng/mL (Chiossone et al., 2007)	20 or 100 ng/mL	5 days
IL-18	100 ng/mL (Agaugué et al., 2008)	100 ng/mL	40 h
IL-15 and IL-2	20 ng/mL and 200 IU/mL respectively	20 ng/mL and 1000 IU/mL respectively	5 days
IL-15 and IL-18	20 ng/mL and 100 ng/mL respectively	20 ng/mL and 100 ng/mL respectively	5 days

2.4 Flow cytometry analysis

2.4.1 Flow cytometry analysis set-up

Before flow cytometry analysis, BD Calibrate beads were ran to accurate the set-up of channels on the FACSCaliburTM machine. Thereafter, four-colour compensation of FL1 (FITC), FL2 (PE), FL3 (PerCP or PE-Cy5) and FL4 (APC) was performed manually and saved for further experimental analysis. Compensation was carried out using PBMC or CBMC stained with single colour and unstained as a negative control. For negative control, voltages of all four channels were adjusted to place the population at the lower left quadrant of the dot plot. Then, a single colour FITC tube containing a positive population (CD16), a single colour PE tube containing a positive population (CD3), a single colour PerCP or PE-Cy5 tube containing a positive population (CD3 or CD56) and a single colour APC tube containing a positive population (CD56) were ran and compensation was adjusted accordingly (FL2 – %FL1, FL1 – %FL2, FL3 – %FL2, FL4 – %FL3 and FL3 – %FL4). Antibodies were titrated prior use for optimal antigenic expression using different dilutions including 1:5, 1:10, 1:20, 1:50, 1:100 and 1:200.

2.4.2 Phenotype of activated NK cells

The expression of surface markers on NK cells was examined using a FACSCalibur™ flow cytometer. NK cells (0.5 to 2.5×10^5 cells) were stained in V bottom Nunc® plates with fluorochrome-conjugated antibodies for 10 min at 4 °C. FACS buffer was used to wash away unbound antibodies by centrifugation at $550 \times g$ for 3 min. Cells were then resuspended in 200 µL FACS buffer. The expression of activation markers, KIRs, lectin-like inhibitory receptors, chemokine receptors and adhesion molecules was assessed on both resting and activated NK cells (Table 2.13). Combinations of monoclonal antibodies used in this study are listed at Table 2.14.

Table 2.13 Surface markers analysed in this study and their significance.

Marker	Significance	FC*	Dil.
CCR7	Lymph-node homing receptor (BD)	PE	1:50
CCR7	Lymph-node homing receptor (R&D)	PE	1:10
CD3	Pan-T cell marker	PerCP	1:20
CD3	Pan-T cell marker	PE	1:17
CD16	FcγIII receptor (ADCC pathway)	FITC	1:10
CD16	FcγIII receptor (ADCC pathway)	APC	1:200
CD56	Pan-NK cell marker	PE-Cy5	1:10
CD56	Pan-NK cell marker	APC	1:10
CD62L	Adhesion molecule (L-selectin)	FITC	1:50
CD69	Activation marker	APC	1:50
CD94	Lectin-like killer inhibitory receptor	FITC	1:100
CD158a	Killer cell Ig-like receptor	FITC	1:5
CD158b	Killer cell Ig-like receptor	FITC	1:10
CXCR3	Lymph-node homing receptor	APC	1:10
NKG2A	Lectin-like killer inhibitory receptor	PE	1:20
NKp44	Natural Cytotoxicity receptor	APC	1:10

FC*= Fluorochrome

Table 2.14 Panel of monoclonal antibodies used to study phenotype of activated NK cells.

FITC	PE	PerCP	PE-Cy5	APC
CD158a	CD3		CD56	CD69
CD158b	CD3		CD56	NKp44
L-selectin	CD3		CD56	CXCR3
CD94	NKG2A	CD3		CD56
CD16	CCR7	CD3		CD56
Annexin V	CD3	7AAD		CD56

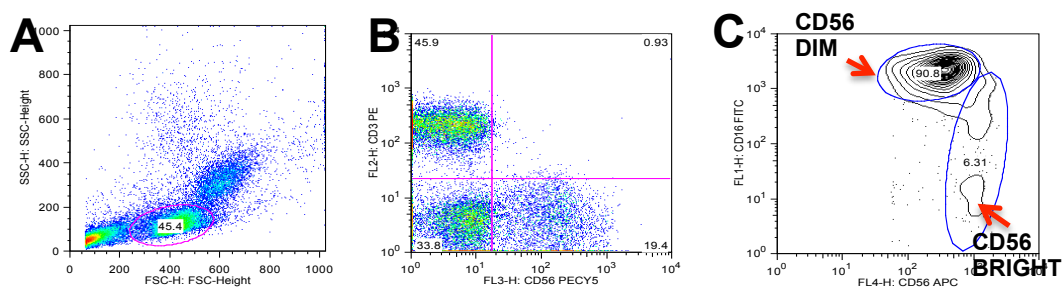
2.4.3 Analysis of cytokine receptor expression

Cytokine receptor expression was investigated using flow cytometry. Mononuclear cells from both PB and CB were washed and stained using titrated monoclonal

antibodies (Table 2.15). The expression of cytokine receptors was analysed on CD56^{bright} CD16^{dim} and CD56^{dim}CD16^{bright} NK cell subsets. Combinations of monoclonal antibodies used for this analysis are listed in Table 2.16.

Table 2.15 Monoclonal antibodies used to analyse the expression of cytokine receptors on NK cell subsets.

Receptor	Alternate name	Description	Dilution used
IL-2R α	CD25	High affinity IL-2 receptor	1:100
IL-2R β	CD122	Intermediate affinity IL-2 receptor	1:20
IL-12R β 1	CD212	Beta-1 subunit, forms heterodimer with Beta-2 required for IL-12 binding	1:20
IL-12R β 2	CD212	Beta-2 subunit, forms heterodimer with Beta-1 required for IL-12 binding	1:50
IL-15R α	-	High affinity IL-15 receptor	1:20
IL-18R α	CD218	High affinity IL-18 receptor	1:100

**Figure 2.2** Gating strategy to identify CD56^{bright} and CD56^{dim} NK cells. The graph shows the gating strategy used to analyse the expression of cytokine receptors by NK cell subsets. A) FSC vs. SSC plot showing the lymphocyte gate. B) Gated NK cells are CD3⁻CD56⁺. C) The expression of each cytokine receptor was assessed on both CD56^{dim}CD16^{bright} and CD56^{bright}CD16^{dim} NK cell subsets.**Table 2.16** Panel of monoclonal antibodies used to study the expression of cytokine receptors on PB and CB NK cells.

FITC	PE	PerCP	PE-Cy5	APC
CD16	CD3		CD56	IL-2R α
CD16	IL-2R β	CD3		CD56
CD16	CD3		CD56	IL-12R β 1
CD16	IL-12R β 2	CD3		CD56
CD16	IL-15R α	CD3		CD56
IL-18R α	CD3		CD56	CD16

2.4.4 Viability of NK cells

NK cell viability was analysed post-incubation with cytokines using 7AAD and Annexin V. Concentrations of 7AAD and Annexin V used were 1/160 and 1/100 respectively.

2.5 Signalling pathway analysis

The BD™ Phosflow Protocol (Harsh alcohol method) was used to study activation of the STAT signalling pathway in NK cells. Phosphorylation of STAT5 and STAT4 in NK cells was assessed at 15 min or two days after cytokine stimulation with IL-2 or IL-12 at the concentrations indicated in table 2.12. NK cells at concentration of 1×10^5 were fixed using 100 μ L pre-warmed BD Cytofix™ Fixation Buffer and permeabilised according to the manufacturers recommendations. Subsequently, NK cells were stained with STAT5 (pY694) PE for those cells stimulated with IL-2 and STAT4 (pY693) PE for those cells stimulated with IL-12 (Wang et al., 2000). Unstimulated NK cells as well as isotypes (PE-Mouse IgG₁ κ , PE-Mouse IgG_{2b}) were included as controls.

2.6 Assessment of NK cell proliferation

PB and CB NK cells were washed and labelled with 2 μ M Carboxyfluorescein succinimidyl ester (CFSE) per 10^6 cells. CFSE was resuspended in DMSO prior to use according to the manufacturers recommendations. NK cells were incubated with CFSE at 37 °C 5 % CO₂ for 10 min. The cells were then washed twice with cold RPMI supplemented with 10 % v/v FBS. CFSE is a light-sensitive chemical; therefore, labelling was carried out in the dark. CFSE labelling was detected at time point 0 using the FL-1 channel on a FACSCalibur™ flow cytometer. CFSE labelled cells were incubated with different cytokines (Table 2.12) and cell proliferation analysed at days 2 and 5. CFSE fluorescence is diluted in successive generations of NK cells and correlates with proliferation.

2.7 Cytokine secretion

The Human T_H1/T_H2 11plex Ready-to-Use FlowCytomix Multiplex Kit (eBioscience) was used to study cytokine secretion by NK cells according to the manufacturers

protocol. Beads coated with antibodies for specific target analytes including human IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF- α and TNF- β were mixed with culture supernatants and secondary biotin-conjugated antibody. Subsequently, samples were labelled with Streptavidin-PE. The purpose of using this method was to perform wide screening of cytokine production by NK cells. Tests were performed using culture supernatants from cytokine-conditioned NK cells (Table 3.12). Before running samples, setup beads were used to create a FlowCytomix protocol and to adjust both voltage and compensation. The final results were analysed using the FlowCytomix 3.0 software (Fig 2.3).

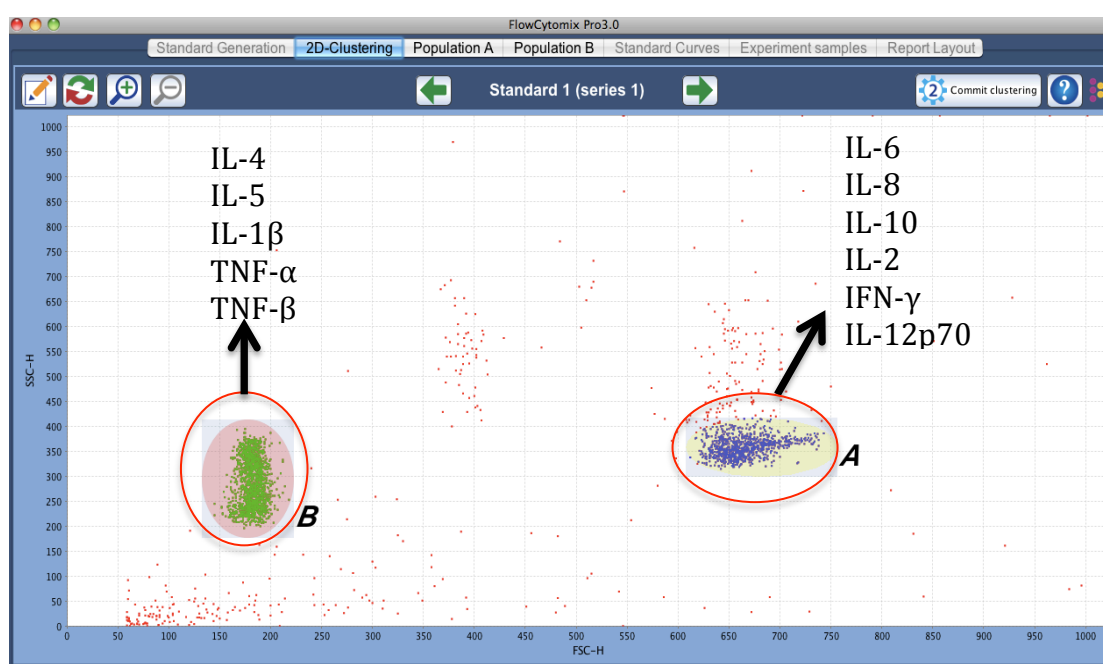


Figure 2.3 Gating strategy using antibody-labelled micro-beads as shown by FlowCytomix software. The two populations indicate the different sizes of antibody coated-beads used to target 11 analytes.

2.8 NK cell cytotoxicity

NK cell cytotoxicity was investigated after 2 and 5 days of activation with cytokines. The target cell line used in this assay was K562. The HLA-deficient leukemic cell line was sub-cultured prior use with 20% v/v FBS supplemented culture medium. Cells were then maintained in 10% v/v FBS culture media and used for the assay when reaching the log phase at concentration of 0.5×10^6 /mL. K562 cells were then washed with PBS and incubated with $^{51}\text{Chromium}$ (^{51}Cr) at a concentration of 100 μCi / 1×10^6

cells at 37 °C, 5 % CO₂, 96 % humidity for 45 min. Subsequently, K562 cells were washed and co-cultured with effector NK cells at different ratios (Effector:target) of 1:1, 5:1 and 10:1 for 4 h at 37 °C (Fig 2.4). Medium alone was used as a negative control and cells lysed with Triton X-100 were used as a positive control. After incubation, plates were centrifuged and 30 µL of supernatant was transferred into LumaPlates and dried overnight. The next day, scintillation liquid was added to each well and the plates were read using a Perkin Elmer 1450 liquid scintillation counter. The percentage of specific lysis was calculated as (experimental release – spontaneous release^{*}) ÷ (maximum release^{**} – spontaneous release) × 100.

Spontaneous release^{*} = negative control.

Maximum release^{**} = positive control.

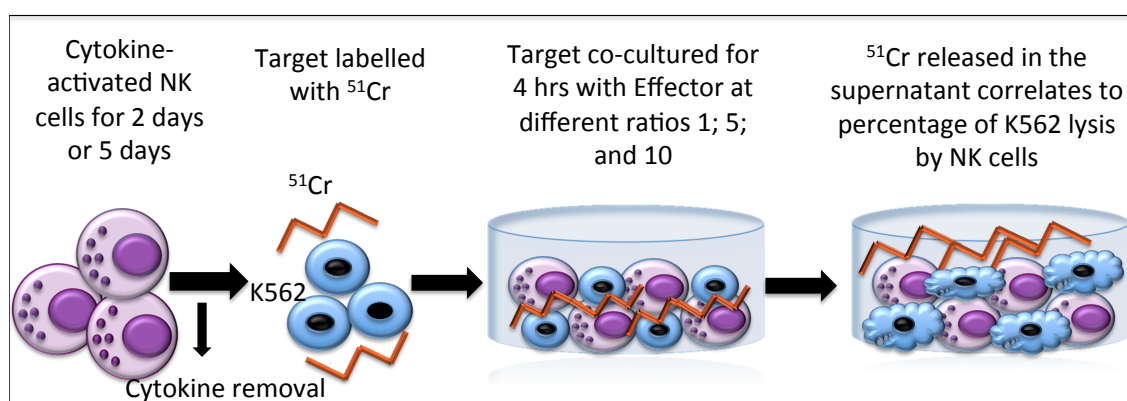


Figure 2.4 Principle of killing assay using ⁵¹Cr-labelled K562 cells.

2.9 Real-time PCR

2.9.1 RNA extraction

The RNeasy mini-kit was used to extract RNA from resting or activated (24 h) NK cells according to the manufacturers recommendations. A NanoDrop-1000 Spectrophotometer was used to measure the concentration as well as the purity of the isolated RNA. RNA was stored at -70 °C until analysed.

Table 2.17 RNA extracted from PB NK cells used for RT-PCR.

Sample No.	#1	#2	#3	#4	#5	Yield	Average
PB resting	16.1	23	22.5			RNA ng/ μ L	20.53
	1.86	2.09	2.15			260/280 ratio	2.03
PB IL-2	35.5	16.6	22.5	12.6	22.8	RNA ng/ μ L	22
	1.92	2.03	2.02	1.89	1.93	260/280 ratio	1.96
PB IL-15	17.2	14.8	20	12.3	11.5	RNA ng/ μ L	15.16
	1.81	2.06	1.87	2.01	1.88	260/280 ratio	1.93
PB IL-12	22	14.9	18.7	15.4	11.6	RNA ng/ μ L	16.52
	1.83	1.66	1.9	1.73	2.3	260/280 ratio	1.88
PB IL-18	14.6	22.9	14.3	12.2	20.7	RNA ng/ μ L	16.94
	1.91	1.78	1.99	1.92	2.3	260/280 ratio	1.98
PB IL-15+IL-2	31.8	20.2	21.8	14.2	13.4	RNA ng/ μ L	20.28
	1.94	1.94	2.19	1.75	1.76	260/280 ratio	1.92
PB IL-15+IL-18	43.4	42.2	9	19.8	18.2	RNA ng/ μ L	26.52
	2.02	2.1	2.19	1.87	2.07	260/280 ratio	2.05

Table 2.18 RNA extracted from CB NK cells used for RT-PCR.

Sample No.	#1	#2	#3	#4	#5	#6	Yield	Average
CB resting	49.8	88.8	129.6				RNA ng/ μ L	89.40
	2.02	1.95	1.94				260/280 ratio	1.97
CB IL-2	94	98.9	85.5				RNA ng/ μ L	92.8
	1.94	1.95	1.94				260/280 ratio	1.94
CB IL-15	28.8	72.8	69.3	25.29	14	32.1	RNA ng/ μ L	40.38
	1.9	1.94	1.97	1.88	1.95	1.93	260/280 ratio	1.93
CB IL-12	65.5	70.5	52.1	18	38.1	61.3	RNA ng/ μ L	50.92
	1.98	2	1.99	2.05	2	1.99	260/280 ratio	2.00
CB IL-18	89.6	98.4	73.8	16.3	67.1	68.1	RNA ng/ μ L	68.88
	1.96	1.92	1.9	1.84	2.05	2.03	260/280 ratio	1.95
CB IL-15+IL-2	65.4	58.1	72	51.2	29.3	37.4	RNA ng/ μ L	52.23
	1.85	1.83	1.9	1.92	1.85	1.9	260/280 ratio	1.88
CB IL-15+IL-18	78.6	52.3	83	46.3	31.2	37.8	RNA ng/ μ L	54.87
	1.86	1.82	1.89	1.84	1.92	1.97	260/280 ratio	1.88

2.9.2 Reverse transcription

Reverse transcription of extracted RNA was performed using a Mastercycler thermocycler (Eppendorf, US). RNA at the concentration of 200 ng was mixed with 2

μL of random primers (1 μg) and 1 μL of 25 μmol dNTPs incubated first for 5 min at 65 $^{\circ}\text{C}$ and then on ice for another 5 min. Afterwards, 5X First-strand buffer, 0.1 M DTT and RNasin inhibitor was added to the reaction and incubated at 25 $^{\circ}\text{C}$ for 10 min followed by an additional 2 min at 42 $^{\circ}\text{C}$. A volume of 1 μL (200 UI) of Superscript reverse transcriptase was added to the reaction and incubated at 42 $^{\circ}\text{C}$ for 50 min followed by an additional 15 min at 70 $^{\circ}\text{C}$. Lastly, 40 μL of RNase free water was added making the volume of cDNA 60 μL that was then ready to use for real-time PCR.

2.9.3 Real-time PCR plate set-up

Primers were mixed, according to the concentrations provided in Table 2.10, with RNase-free water and precision SYBRgreen MasterMix. A volume of 15 μL of primer-masternmix was added to MicroAmp® optical 96-well reaction plates. Then, 5 μL of cDNA was added to the wells and plates were covered with MicroAmp® Adhesive film. For each sample, three reference housekeeping genes were used including Homo sapiens Actin-beta (ACTB), Homo sapiens $\beta 2$ microglobulin ($\beta 2\text{M}$) and Homo sapiens Ubiquitin (UBC). Reference gene primer sequences were provided by PrimerDesign and assayed at a concentration of 300 nM. Target genes and reference genes were processed in triplicates and ran on a 7500 Real Time PCR AB Biosystem machine at conditions the following conditions; 50 $^{\circ}\text{C}$ for 2 min, 95 $^{\circ}\text{C}$ for 10 min, 40 cycles for 15 sec at 95 $^{\circ}\text{C}$, 60 $^{\circ}\text{C}$ for 1 min, 95 $^{\circ}\text{C}$ for 15 sec, 60 $^{\circ}\text{C}$ for 1 min and 95 $^{\circ}\text{C}$ for 15 sec. Sterile water was used as a negative control to ensure pure primer amplification. mRNA quantity of target genes was normalised against the average reference gene quantity.

2.10 Migration assays

Trans-well migration assays were carried out to test the ability of NK cells to migrate in response to chemokines. Trans-well medium (Table 2.5) supplemented with 250 ng/mL CCL19/CCL21 or CXCL10/CXCL11 was added to the lower chamber of 96-well HTS Trans-well plates supported with a 5.0 μm polycarbonate membrane (Table 2.19). Subsequently, resting or activated NK cells ($0.1\text{-}0.25 \times 10^6$) from PB and CB were placed into the upper chamber of the trans-well plate. Medium only instead of chemokines was included to assess spontaneous migration of NK cells. The plates were incubated for 3 h at 37 $^{\circ}\text{C}$. Cells that migrated to the lower chamber were collected and

enumerated by flow cytometry. The percentage of migration was calculated using the following formula: % of migration = (No of migrated cells* ÷ Positive control**) \times 100.

No of migrated cells* = Number of NK cells migrated in response to CCL/CXCL.

Positive control** = Total number of cells placed in the lower chamber as a control.

Table 2.19 Chemokines used for migration study

Chemokine	Alternative names	Ligand	Company
Human recombinant CCL19	MIP-3 β	CCR7	Biolegend
Human recombinant CCL21	6CKine	CCR7	Biolegend
Human recombinant CXCL10	IP-10	CXCR3	Biolegend
Human recombinant CXCL11	ITAC	CXCR3	Biolegend

2.11 Cytokine-induced memory-like NK cells

To generate cytokine-induced memory-like NK cells we followed the approach of Romee and colleagues (Romee et al., 2012). For this, freshly isolated NK cells (5×10^6) from PB or CB were pre-activated with 10 ng/mL IL-12, 50 ng/mL IL-18 and 1 ng/mL IL-15 or only 1 ng/mL IL-15 as a control for 16 h. Cells were washed three times to ensure removal of cytokines and cultured in RPMI supplemented with 10 % v/v AB serum and 1 ng/mL IL-15 to support survival of NK cells. NK cells were cultured in 96 U bottom plates and the medium was changed by hemi-depletion every 48 h. At days 7 and 21, cells were washed and restimulated with 10 ng/mL IL-12 and 100 ng/mL IL-15 for 6 h (Fig 2.6). Supernatants were collected and kept at -70 °C to be assayed for IFN- γ production by multiplex assay as described above (section 2.3.8).

To assess intracellular IFN- γ expression, GolgiStop was added to NK cells after restimulation with IL-15 and IL-12 at days 7, 14 and 21 to inhibit transportation of intracellular IFN- γ . Thereafter, NK cells were fixed and permeabilised using Cytofix/Cytoperm buffer according to the manufacturers recommendations. Staining with monoclonal antibodies against IFN- γ following permeabilization was then performed. An isotype control was used for each condition (IgG1 κ FITC, BD Biosciences) (Table 2.20). To assess IFN- γ mRNA levels, real-time PCR was performed at day 21 as stated previously.

Table 2.20 Panel of monoclonal antibodies used to study intracellular IFN- γ expression by cytokine-induced memory-like NK cells.

FITC	PE	APC
IgG1 $_{\kappa}$	CD3	CD56
IFN- γ	CD3	CD56

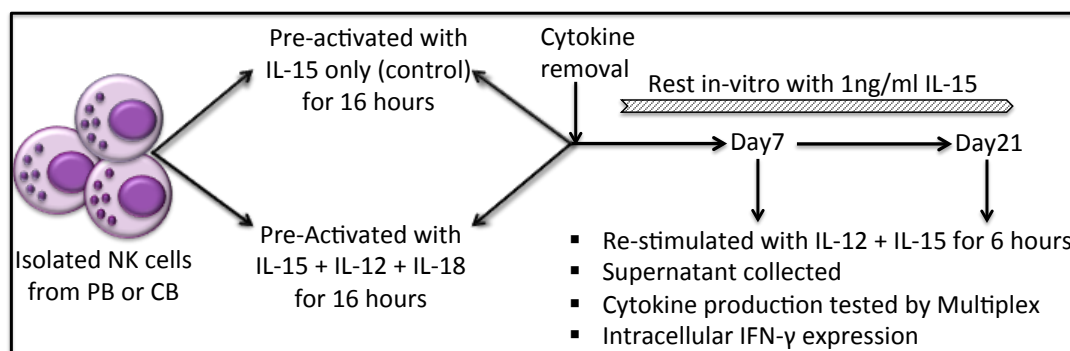


Figure 2.6 Protocol for the generation of cytokine-induced memory-like NK cells. “This figure has been reproduced from an open access article under creative commons attribution license” (Romee et al., 2014).

2.12 Statistical analysis

Statistical comparisons were performed with GraphPad Prism software using unpaired t-tests. Results are presented as Mean \pm SD and p values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered statistically significant.

Chapter 3: Phenotype of Cytokine-activated NK Cells

3.1 Introduction

Resting CB NK cells exhibit unique phenotypic characteristics in comparison to PB NK cells that are suggestive of a certain degree of immaturity of these cells. It has been reported that resting CB NK cells exhibit low cytotoxicity against target cells without prior activation (Dalle et al., 2005, Gaddy et al., 1995, Luevano et al., 2012a, Wang et al., 2007). However, when activated with high doses of IL-2, effector functions of CB NK cells are enhanced, but are still lower than for IL-2 activated PB NK cells (Condiotti et al., 2001, Luevano et al., 2012a). The phenotype of CB NK cells after activation has not been well characterised and therefore it is key to better understand the characteristics of these cells after cytokine activation in comparison to PB NK cells.

NK cells express at least one inhibitory receptor for self-MHC to prevent killing of healthy cells (Moretta et al., 1996). In particular, KIR (CD158a and CD158b) and CD94/NKG2A are key in regulating NK cell functions, as their expression correlates with the degree of maturity of NK cells (Freud and Caligiuri, 2006). In this context, it has been shown that resting CB NK cells exhibit low KIR expression and high NKG2A levels suggesting that CB NK cells are immature and unlicensed (Dalle et al., 2005, Luevano et al., 2012a, Wang et al., 2007). It has been reported that murine NK cells acquire a licensed phenotype after transfer into a MHC class I sufficient host, or after stimulation with IL-12 and IL-18 (Kim et al., 2005). However, cytokine activation of human NK cells does not induce responsiveness of KIR⁺ NK cells (Anfossi et al., 2006). Although CB NK cells are not licensed as compared to PB NK cells, they seem to become competent upon cytokine activation. Wang *et al.* correlated poor cytotoxicity of CB NK cells with high expression of the inhibitory receptor NKG2A (Wang et al., 2007). Whether cytokine activation impacts on the expression of inhibitory receptors by CB NK cells remains to be investigated.

CD69 is an early activation marker expressed by activated lymphocytes, including NK cells (Hara et al., 1986, Lanier et al., 1988). Upregulation of CD69 by NK cells has been reported to trigger NK cell cytotoxicity (Moretta et al., 1991). Moreover, CD69

expression has also been implicated in other functions of NK cells including proliferation, cytokine secretion and upregulation of adhesion molecules (Borrego et al., 1999). NKp44 is a receptor that belongs to the NCR family. Importantly, NKp44 is the only NCR that is specifically upregulated by activated NK cells (Vitale et al., 1998) and NKp44 upregulation by activated NK cells mediates tumour cell lysis (Vitale et al., 1998). CB NK cells express CD69 in response to IL-2 (Condiotti et al., 2001), IL-12, IL-15 (Lin et al., 2000) or IL-18 (Nomura et al., 2001) stimulation. Moreover, NKp44 expression by CB NK cells can be induced after stimulation with IL-2 (Xing et al., 2010) or IL-15 (Tang et al., 2008). However, a complete analysis, as well as a comparison of the effects of different cytokines on promoting activation of CB NK cells is still required, as some of these studies have been carried out using mononuclear cells and not isolated NK cells.

NK cells express a variety of receptors that allow them to respond to cytokines released by other immune cells (Caligiuri et al., 1990, Kunikata et al., 1998, Pillet et al., 2009, Wang et al., 2000). Cytokine receptor expression is also known to be associated with specific NK cell maturation stages. According to the NK cell development model proposed by Freud *et al.*, less differentiated NK cells (CD56^{bright}) express high levels of IL-2R α , whereas mature NK cells (CD56^{dim}) have scarce expression of this receptor (Freud and Caligiuri, 2006). Our group and others have previously shown that CB NK cells express IL-2R β , but have low expression of IL-2R α (Dalle et al., 2005, Luevano et al., 2012a). Yet, the expression of other cytokine receptors by the CD56^{bright} and CD56^{dim} CB NK cell subsets remains to be investigated.

Cytokine activation could represent an approach to induce fully functional CB NK cells. It has been suggested that the phenotype of NK cells correlates with function (Costello et al., 2002). Therefore, the phenotype of CB and PB NK cells following cytokine activation was compared by analysing surface expression of CD69, CD94, NKG2A, NKp44 and KIR. Upregulation of activating receptor expression was used to assess NK cell activation, and to decide which cytokine should be used for optimal activation of CB NK cells. Overall, this chapter discusses the effect of cytokine activation on the phenotype of CB NK cells in comparison to PB NK cells.

3.2 Results

3.2.1 Cytokine induced activation of CB and PB NK cells

3.2.1.1 Cytokine activation induces CD69 expression by PB and CB NK cells

As upregulation of CD69 results in triggering of NK cell cytotoxicity (Moretta et al., 1991), the expression of CD69 following cytokine activation was analysed for PB and CB NK cells. Resting PB and CB NK cells expressed low levels of CD69 (11.67 ± 2.33 % and 6.333 ± 1.2 0% respectively) (Fig 3.1). Following cytokine activation, CD69 expression was differentially upregulated by PB and CB NK cells depending on the cytokine used (Fig 3.1A). CB NK cells activated with IL-2 exhibited significantly less CD69 expression (43.63 ± 13.94 %) than PB NK cells (82.05 ± 5.69 %) ($p = 0.0434$) (Fig 3.1B). Conversely, CB NK cells activated with IL-12 showed higher upregulation of CD69 (90.83 ± 4.40 %) than PB NK cells (67.28 ± 3.52 %) ($p = 0.0058$) (Fig 3.1B). Activation using IL-15 or IL-18 induced comparable upregulation of CD69 for both PB and CB NK cells (Fig 3.1B). CD69 expression was not further upregulated by higher doses of IL-12 (Fig 3.2A) or IL-15 (Fig 3.2B).

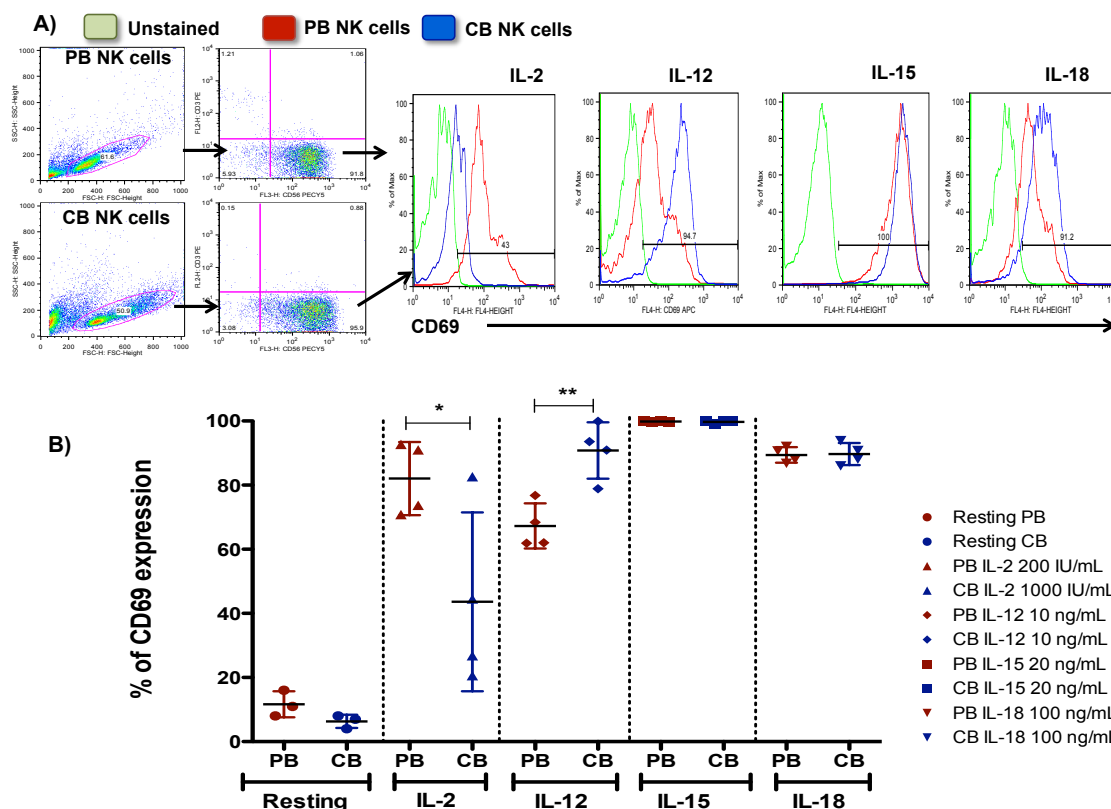


Figure 3.1 CD69 upregulation by cytokine-activated PB and CB NK cells. A) Flow cytometry histograms showing CD69 expression for representative samples of PB and CB NK cells activated with cytokines. B) Comparison of CD69 expression between resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of CD69 was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*) and $p < 0.01$ (**).

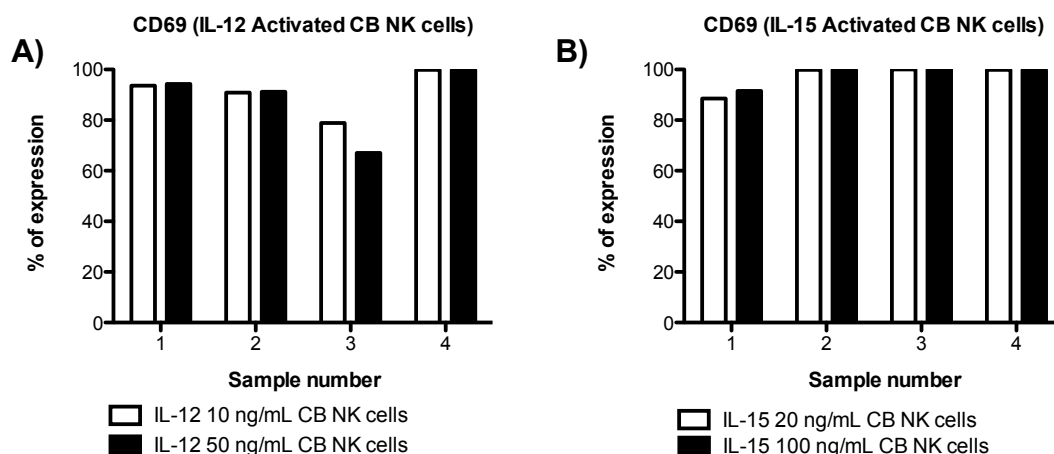


Figure 3.2 Upregulation of CD69 by CB NK cells following stimulation with IL-12 and IL-15. A) CD69 expression by CB NK cells activated with 10 ng/mL or 50 ng/mL IL-12, N=4. B) CD69 expression by CB NK cells activated with 20 ng/mL or 100 ng/mL IL-15, N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

3.2.1.2 Cytokine activation induces NKp44 expression by PB and CB NK cells

NKp44 is a NCR that is only expressed by activated NK cells (Moretta et al., 2000). Here the induction of NKp44 expression on PB and CB NK cells following cytokine activation was analysed. Notably, the NKp44 expression pattern was similar to the expression pattern observed for CD69 on NK cells (Fig 3.3A). IL-2 activated CB NK cells showed significantly less upregulation of NKp44 (50.28 ± 8.78 %) than PB NK cells (17.28 ± 4.72 %) ($p = 0.0163$) (Fig 3.3B). CB NK cells responded better to stimulation with IL-12, showing higher upregulation of NKp44 (68.93 ± 9.83 %) than PB NK cells (34.45 ± 2.00 %) ($p = 0.0139$) (Fig 3.3B). NKp44 expression was higher following stimulation with IL-15 than IL-18 for both PB and CB NK cells (Fig 3.3B). NKp44 upregulation was neither enhanced nor decreased by higher doses of IL-12 (Fig 3.4A) or IL-15 (Fig 3.4B).

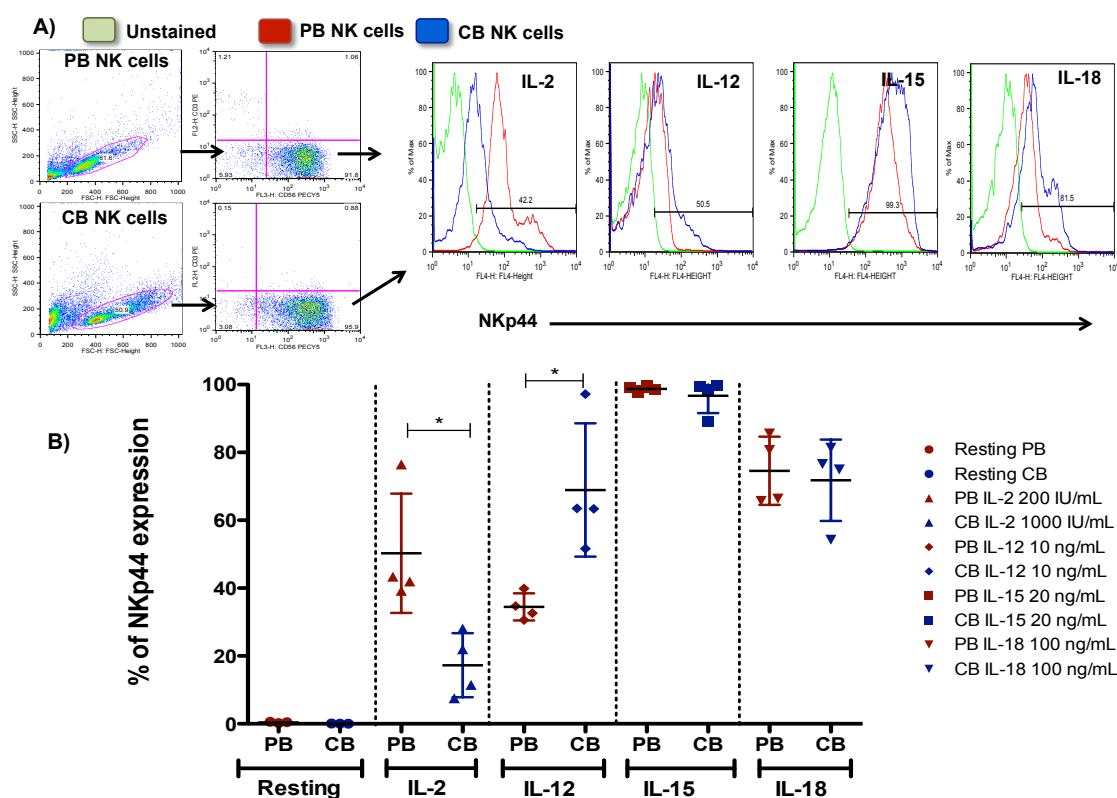


Figure 3.3 NKp44 upregulation by cytokine-activated PB and CB NK cells. A) Flow cytometry histograms showing NKp44 expression for representative samples of PB and CB NK cells activated with cytokines. B) Comparison of NKp44 expression by resting and cytokine-activated NK cells from PB and CB. Analysis was performed on purified NK cells and the expression of NKp44 was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*).

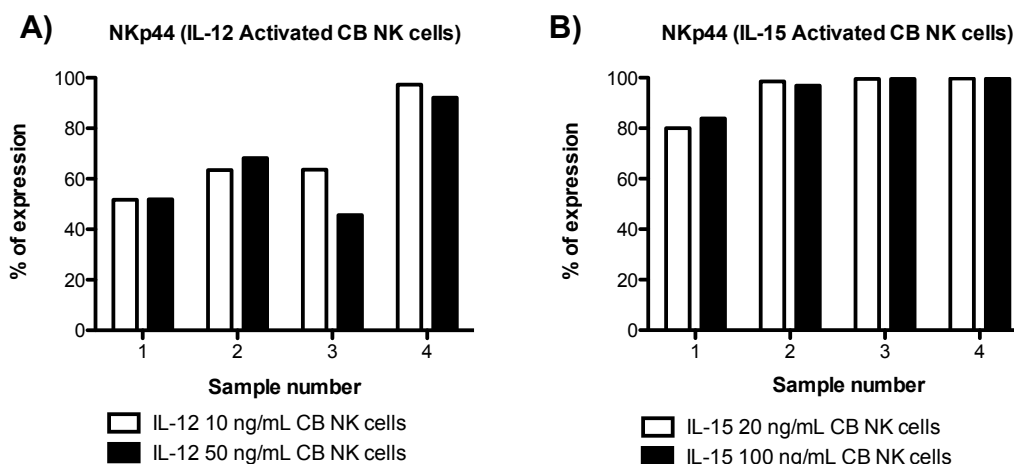


Figure 3.4 NKp44 expression by PB and CB NK cells after stimulation with IL-12 and IL-15. A) Upregulation of NKp44 expression by CB NK cells activated with 10 ng/mL or 50 ng/mL IL-12, N=4. B) Upregulation of NKp44 expression by CB NK cells activated with 20 ng/mL or 100 ng/mL IL-15, N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

3.2.2 Cytokine activation does not impact on killer immunoglobulin-like receptor expression by CB and PB NK cells

NK cells at late stages of differentiation acquire the expression of KIR, hence, mature NK cells are characterised as $CD56^{\text{dim}}KIR^+$ (Freud and Caligiuri, 2006). It has previously been shown that CB NK cells express less KIR than PB NK cells, which was suggestive of CB NK cells being less mature (Luevano et al., 2012a). However, it was not known whether cytokine activation could promote KIR expression by NK cells. Herein, surface expression of KIR by PB and CB NK cells following cytokine stimulation was assessed. Variable expression of CD158a (KIR2DL1) was noted for PB NK cells and CB NK cells with a trend towards lower CD158a expression by CB NK cells in general, regardless of cytokine stimulation (Fig 3.5A, B). Increasing doses of IL-12 (Fig 3.6A) or IL-15 (Fig 3.6B) had no effect on CD158a expression by NK cells. Similarly, expression of CD158b (KIR2DL2/3) was significantly lower for both resting and cytokine-activated CB NK cells than PB NK cells (Fig 3.7). Higher doses of IL-12 or IL-15 did not increase CD158b expression by NK cells (Fig 3.8).

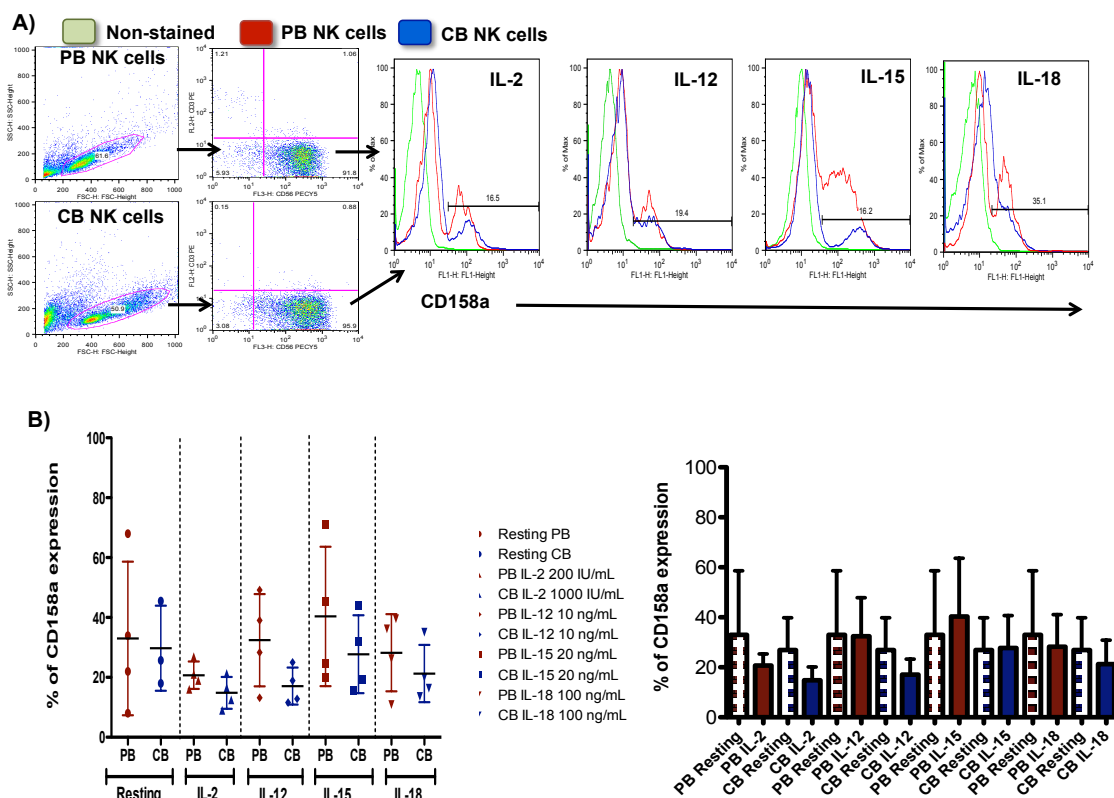


Figure 3.5 Analysis of CD158a (KIR2DL1) expression by resting and cytokine-activated CB and PB NK cells. A) Representative flow cytometry histograms showing CD158a expression by cytokine-activated PB and CB NK cells from different donors. B) Comparison of percent CD158a expression by resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of CD158a was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4.

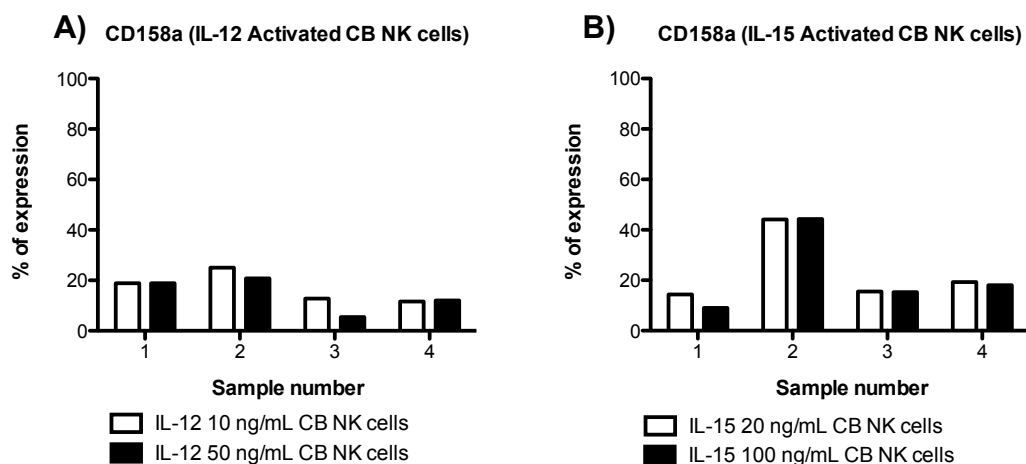


Figure 3.6 CD158a expression by CB NK cells activated with IL-12 and IL-15. A) CD158a expression by CB NK cells activated with IL-12 at 10 ng/mL or 50 ng/mL. B) CD158a expression by CB NK cells activated with IL-15 at 20 ng/mL or 100 ng/mL. N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

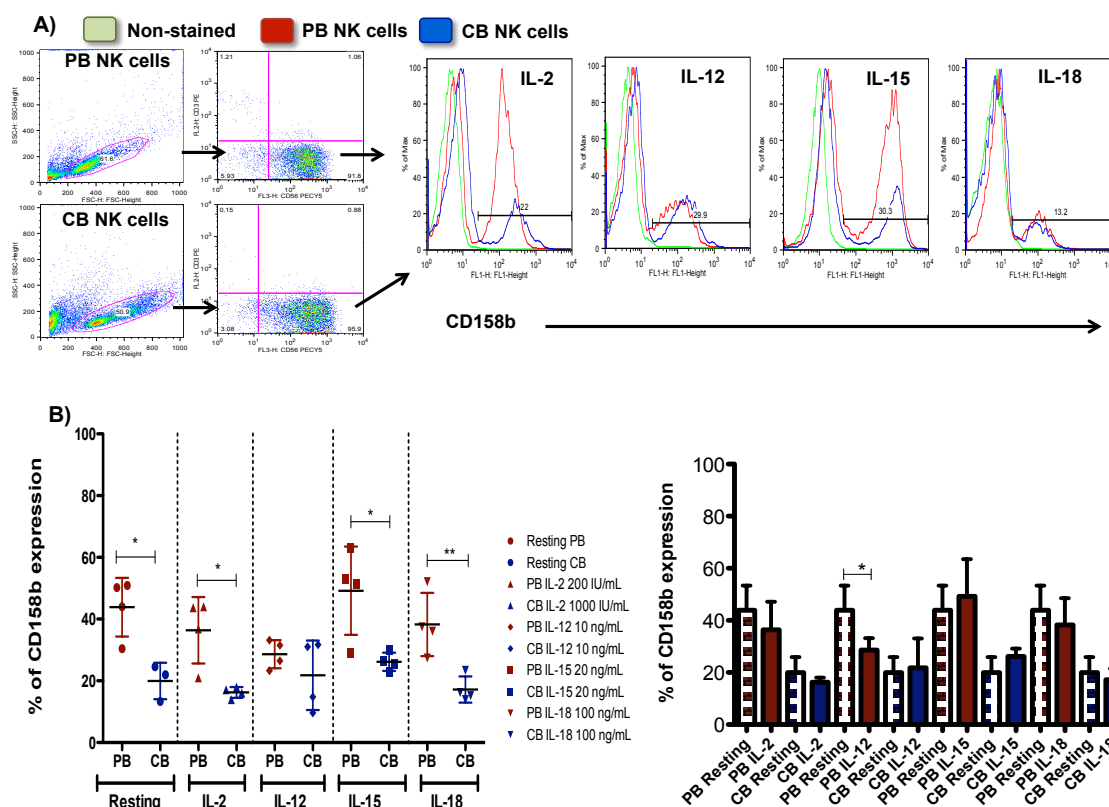


Figure 3.7 CD158b (KIR2DL2/3) expression by resting and cytokine-activated PB and CB NK cells. A) Representative flow cytometry histograms showing the expression of CD158a by cytokine-activated PB and CB NK cells from different donors. B) CD158b expression by resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of CD158b was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, p values of <0.05 (*) and p <0.01 (**).

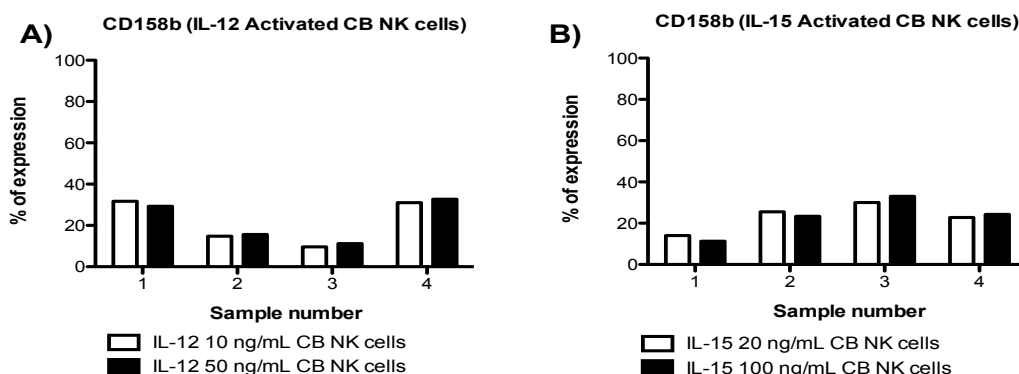


Figure 3.8 CD158b expression by CB NK cells upon activation with IL-12 and IL-15. A) Expression of CD158a by CB NK cells activated with 10 ng/mL or 50 ng/mL IL-12. B) CD158b expression by CB NK cells activated with 20 ng/mL or 100 ng/mL of IL-15. N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

3.2.3 CB NK cells exhibit higher expression of lectin-like inhibitory receptors than PB NK cells, regardless of the cytokine used for activation

NKG2A is an inhibitory receptor expressed by NK cells that binds to non-classical HLA-E expressed by target cells (Braud et al., 1998). It has been shown that immature NK cells are characterised by high expression of NKG2A and low expression of KIR (Freud and Caligiuri, 2006). Accordingly, it is thought that higher expression of NKG2A by CB NK cells may affect their cytotoxicity (Wang et al., 2007). NKG2A is expressed by NK cells as a heterodimer, coupled with CD94 (CD94/NKG2A) (Lazetic et al., 1996). Therefore, the expression of CD94 and NKG2A by CB NK cells and PB NK cells following cytokine activation was compared in order to assess whether cytokine stimulation alters the expression of inhibitory receptors by CB NK cells. A trend towards higher CD94/NKG2A expression by resting or cytokine-activated CB NK cells was observed when compared to PB NK cells (Fig 3.9A, Fig 3.11A). CD94 expression was significantly higher for resting CB NK cells (84.27 ± 1.53 %) as compared to resting PB NK cells (49.75 ± 7.51 %) ($p = 0.0121$) (Fig 3.9B). Consistently, resting CB NK cells exhibited significantly higher NKG2A expression (75.70 ± 1.99 %) than resting PB NK cells (44.40 ± 6.05 %), ($p = 0.0080$) (Fig 3.9B). CD94/NKG2A expression by NK cells was not affected by increasing doses of IL-12 or IL-15 (Fig 3.10 and 3.12).

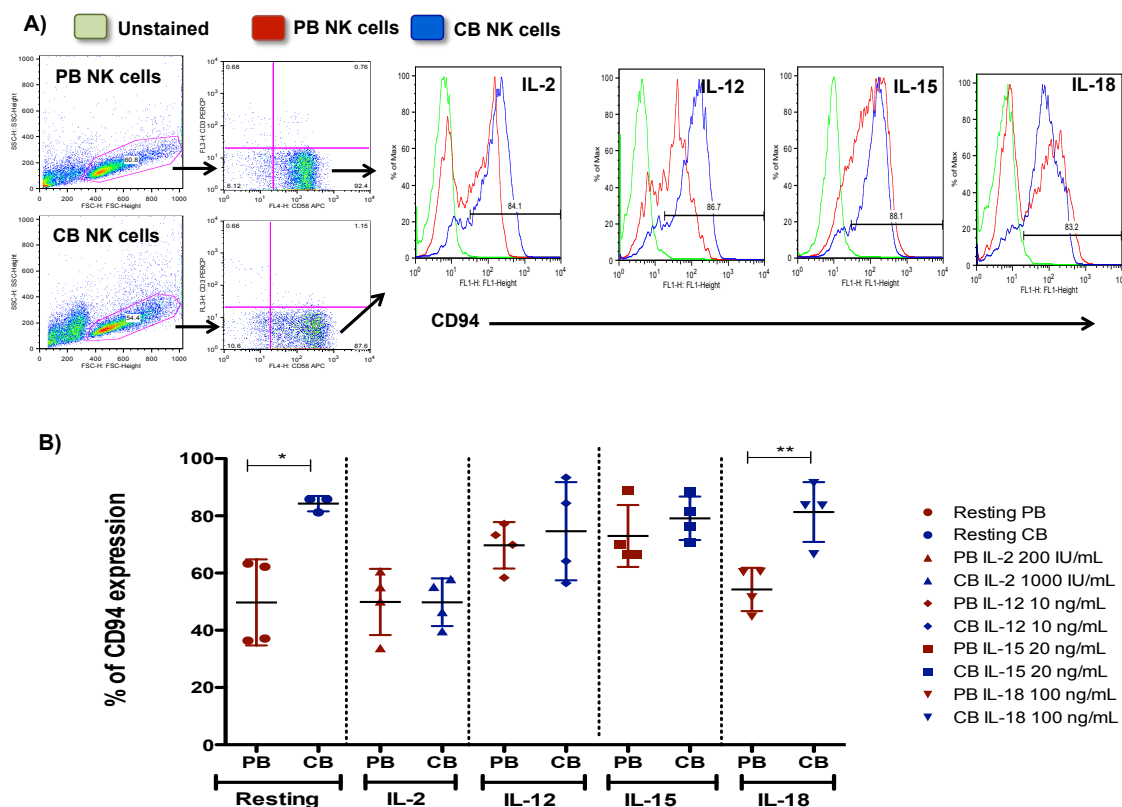


Figure 3.9 CD94 expression by PB and CB NK cells after cytokine activation. A) Flow cytometry histograms showing CD94 expression upon activation of PB and CB NK cells with cytokines. B) Comparison of CD94 expression by resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of CD94 was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*) and $p < 0.01$ (**).

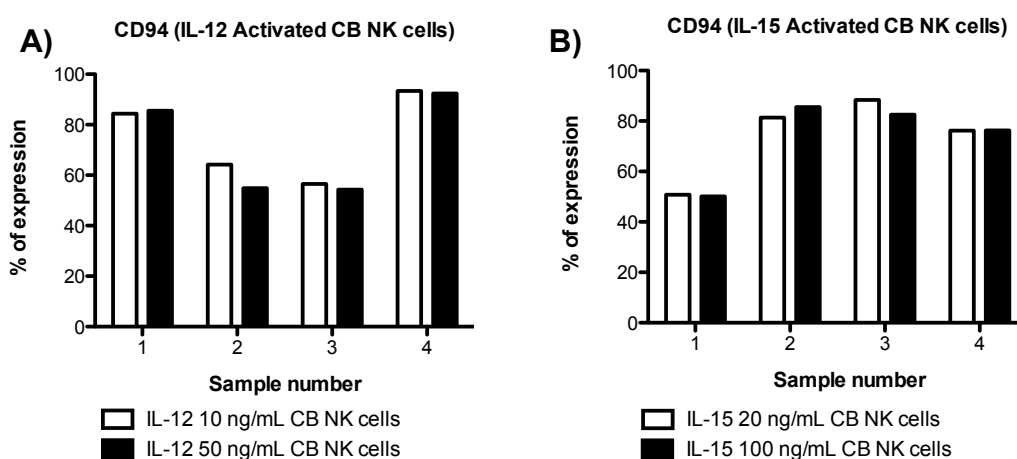


Figure 3.10 CD94 expression by CB NK cells upon activation with IL-12 and IL-15. A) CD94 expression by CB NK cells activated with IL-12 at 10 ng/mL or 50 ng/mL. B) CD94 expression by CB NK cells activated with 20 ng/mL or 100 ng/mL IL-15. N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

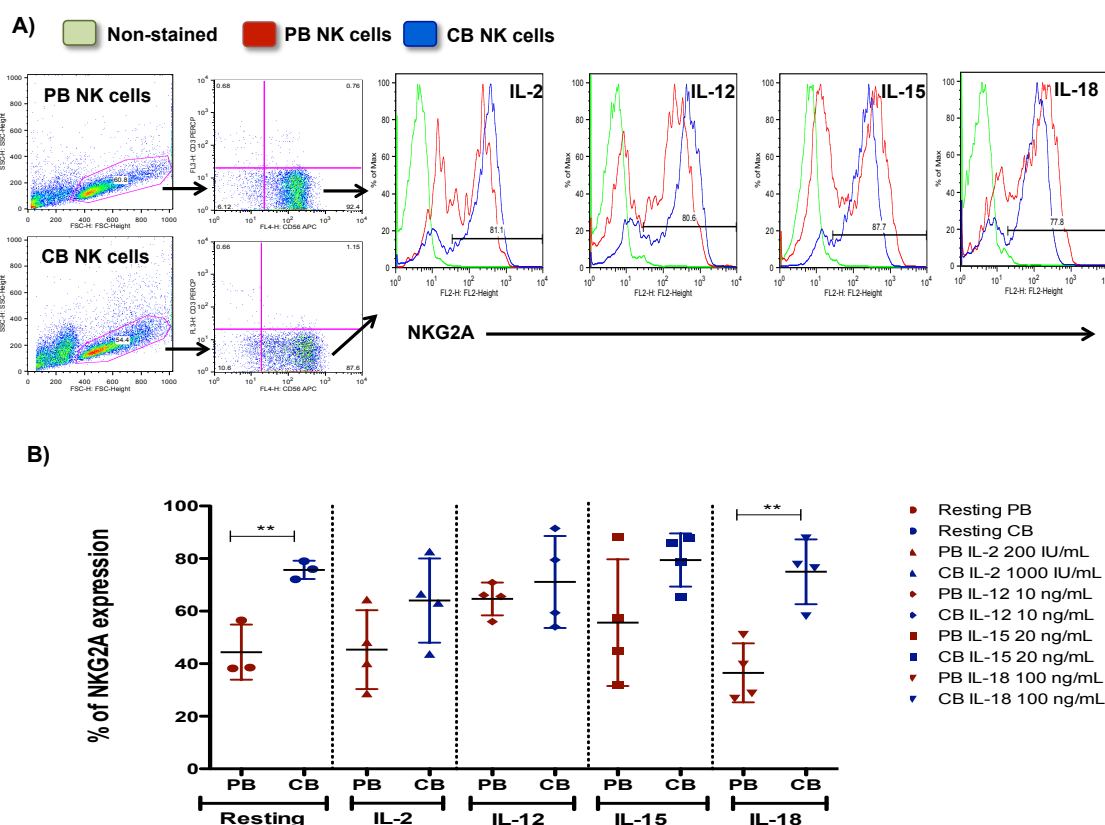


Figure 3.11 NKG2A expression by PB and CB NK cells after cytokine activation. A) Flow cytometry histograms showing NKG2A expression upon activation of PB and CB NK cells with cytokines. B) Comparison of NKG2A expression by resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of NKG2A was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. $N=4$, $p < 0.05$ (*) and $p < 0.01$ (**).

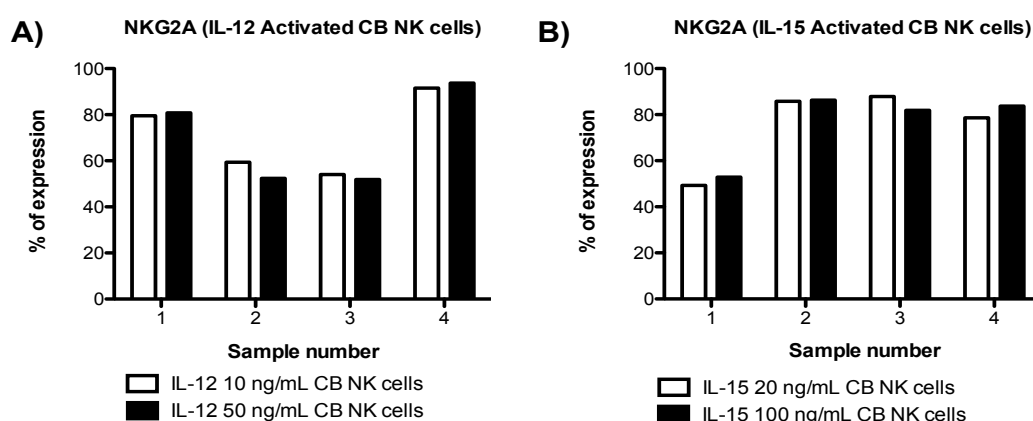


Figure 3.12 NKG2A expression by CB NK cells upon activation with different doses of IL-12 and IL-15. A) NKG2A expression by CB NK cells activated with IL-12 at 10 ng/mL or 50 ng/mL. B) NKG2A expression by CB NK cells activated with 20 ng/mL or 100 ng/mL of IL-15. $N=4$. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

3.2.4 CB NK cells express lower levels of specific cytokine receptors than PB NK cells

Cytokine receptor expression was analysed to determine whether expression correlates with the response of PB and CB NK cells to cytokines. Differential expression of cytokine receptors by NK cell subsets has been previously reported, therefore the expression of cytokine receptors was assessed for both CD56^{bright} and CD56^{dim} NK cells (Caligiuri et al., 1990, Kunikata et al., 1998, Voss et al., 1992). CB CD56^{bright} NK cells exhibited comparable levels of IL-2R α and IL-2R β to PB CD56^{bright} NK cells (Fig 3.13A, E). However, CB CD56^{dim} NK cells expressed significantly less IL-2R α (68.28 ± 2.41 %) than PB CD56^{dim} NK cells (87.86 ± 0.94 %) ($p < 0.0001$) (Fig 3.14A, E). CB NK cell subsets exhibited significantly lower levels of IL-12R β 1 expression (92.00 ± 1.65 % by CD56^{bright} NK cells and 70.81 ± 5.86 % by CD56^{dim} NK cells), as compared to PB CD56^{bright} and CD56^{dim} NK cells (97.78 ± 1.18 % and 91.92 ± 1.28 % respectively) ($p < 0.05$) (3.13 B and E, 3.14 B and E). Conversely, CB NK cell subsets showed slightly higher expression of IL-12R β 2 than PB NK cells (Fig 3.13 B, E and Fig 3.14 B, E). IL-15R α expression was higher on CD56^{bright} CB NK cells (12.88 ± 2.02 %) in comparison to PB CD56^{bright} NK cells (6.05 ± 1.21 %) ($p = 0.0214$) (Fig 3.13 C, E), whereas CD56^{dim} CB NK cells showed equivalent IL-15R α expression to PB CD56^{dim} NK cells (Fig 3.14 C, E). CD56^{bright} CB NK cells expressed significantly less IL-18R α than CD56^{bright} PB NK cells (94.25 ± 1.95 % and 69.99 ± 6.24 % respectively) ($p = 0.0070$) (Fig 3.13 D, E). Similarly, CD56^{dim} CB NK cells showed lower levels of IL-18R α (34.18 ± 4.95 %) than CD56^{dim} PB NK cells (56.80 ± 5.19 %) ($p = 0.0091$) (Fig 3.14 D, E). Overall, PB NK cells express higher levels of IL-2R α , IL-12R β 1 and IL-18R α than CB NK cells, whereas CD56^{bright} CB NK cells express higher levels of IL-15R α than CD56^{bright} PB NK cells.

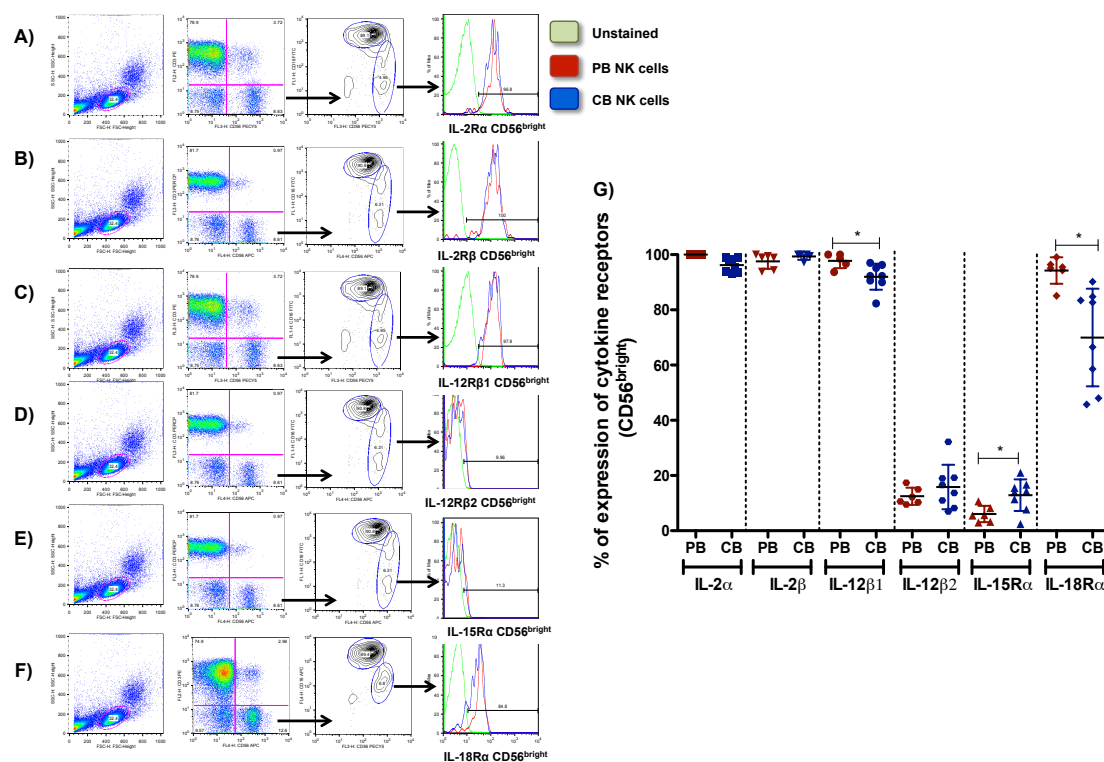


Figure 3.13 Cytokine receptor expression by CD56^{bright} CB and PB NK cells. Gates were adjusted on CD56^{bright} NK cells. Flow cytometric histograms show the expression of IL-2 receptor α (A) and β (B), IL-12 receptor β 1 (C) and β 2 (D), IL-15 receptor α (E) and IL-18 receptor α (F) by PB and CB CD56^{bright} NK cells. G) Comparison of cytokine receptor expression between PB and CB CD56^{bright} NK cells. This analysis was performed on resting lymphocytes. The combination of monoclonal antibodies used in this analysis is shown in Table 2.16. Results are presented as Mean \pm SD. PB N=6, CB N=8, (*) $p < 0.05$.

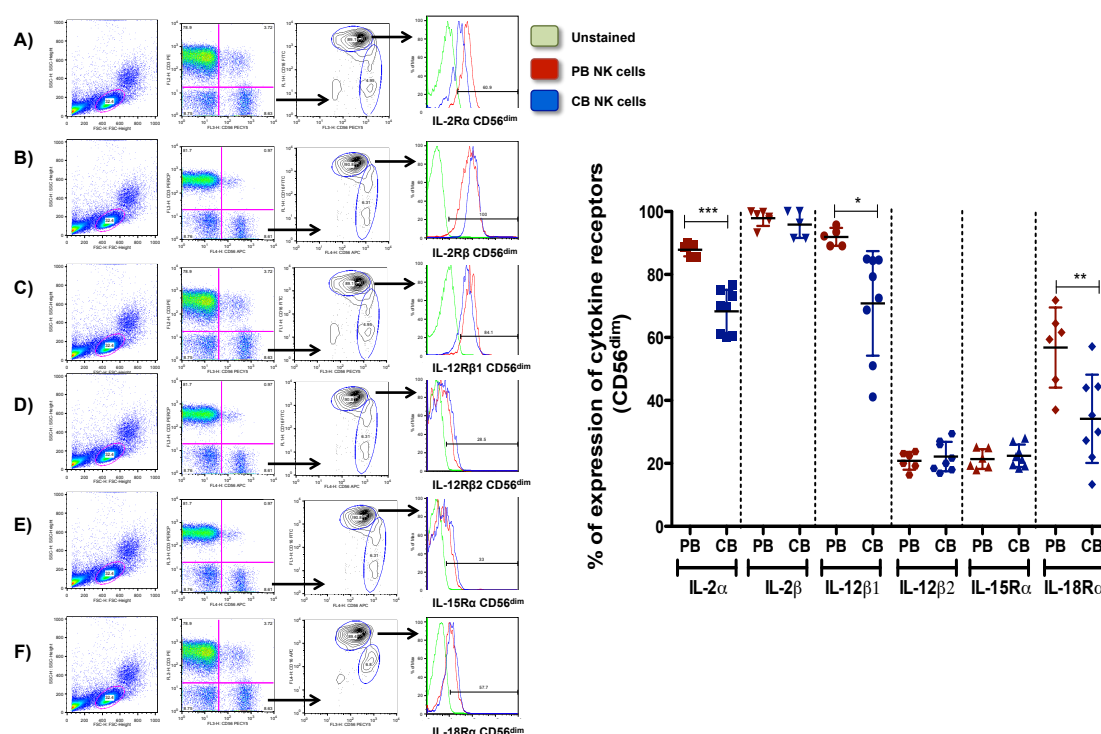


Figure 3.14 Cytokine receptor expression by CD56^{dim} CB and PB NK cells. Gates were adjusted on CD56^{dim} NK cells. Flow cytometric histograms show the expression of IL-2 receptor α (A) and β (B), IL-12 receptor β1 (C) and β2 (D), IL-15 receptor α (E) and IL-18 receptor α (F) by PB and CB CD56^{dim} NK cells. G) Comparison of cytokine receptor expression between PB and CB CD56^{dim} NK cells. This analysis was performed on resting lymphocytes. The combination of monoclonal antibodies used in this analysis is shown in Table 2.16. Results are presented as Mean ± SD. PB N=6, CB N=8, (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

3.2.5 Stimulation of CB NK cells with IL-2 induced less STAT5 phosphorylation than stimulation of PB NK cells

As I observed less activation of CB NK cells following stimulation with IL-2, signalling downstream of this receptor was compared for CB and PB NK cells after stimulation with IL-2. Binding of IL-2 to its receptor initiates phosphorylation of the signal transducer and activator of transcription 5 (STAT5), which is key for NK cell activation, proliferation and survival (Becknell and Caligiuri, 2005, Carson et al., 1994). Intracellular expression of phosphorylated (p)-STAT5 was therefore assessed after stimulation of NK cells with IL-2 for 15 min or 48 h. The latter time point was selected because optimal induction of IL-2Rα expression is reported to occur after approximately 48 h of activation and may result in an increased p-STAT5 expression (Johnston et al., 1995, Pillet et al., 2009). STAT5 was significantly less phosphorylated in CB NK cells compared to PB NK cells (Fig 3.15 A). After 15 min of IL-2

stimulation, the percentage of p-STAT5 expression by PB and CB NK cells was $81.94 \pm 7.6\%$ and $45.80 \pm 5.86\%$ respectively ($p = 0.0057$). At day two, the percentage of p-STAT5 expression by PB NK cells was $89.33 \pm 2.81\%$, whereas it was $43.00 \pm 5.82\%$ for CB NK cells ($p < 0.0001$) (Fig 3.15 B). Lower density of p-STAT5 expression (MFI) was observed for CB NK cells after 15 min and two days of activation (p value < 0.0001 and 0.0006 respectively) (Fig 3.15 C).

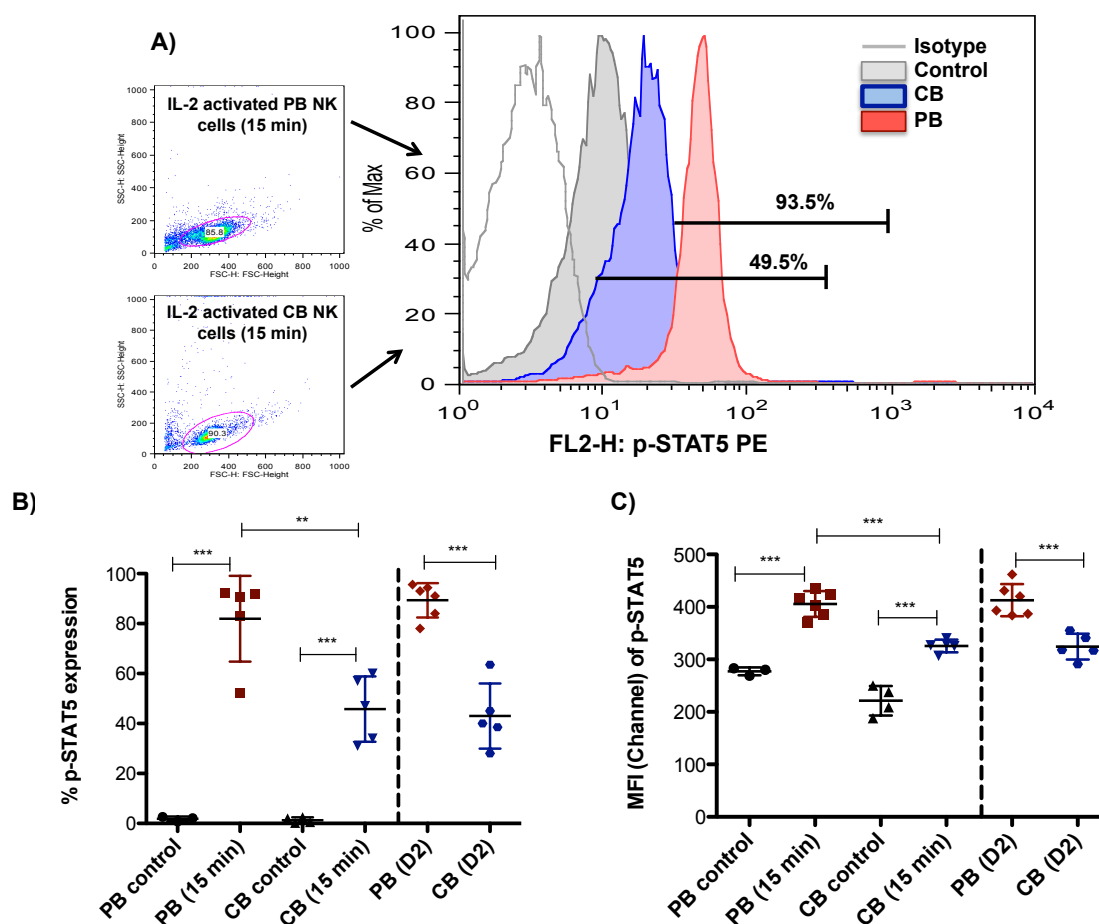


Figure 3.15 Analysis of phosphorylated STAT5 expression by cytokine-activated CB and PB NK cells. A) Flow cytometric analysis of the relative phosphorylation of STAT5 (p-STAT5) after 15 min stimulation of PB and CB NK cells with IL-2. B) Mean percentage of expression of p-STAT5 after 15 min of stimulation with IL-2. C) Density (MFI) of p-STAT5 expression after stimulation with IL-2 for 15 min or 2 days for PB and CB NK cells. Analysis was performed on purified NK cells stimulated with IL-2 for 15 min or 2 days. Results are presented as Mean \pm SD. Control = Unstimulated NK cells. PB N=5, CB N=5, Control N=3. $p < 0.01$ (**), and $p < 0.01$ (***).

Higher expression of CD69 and NKp44 was observed for CB NK cells activated with IL-12 as compared to PB NK cells activated with the same cytokine. Therefore,

activation of signalling molecules downstream of the IL-12 receptor were also compared for CB and PB NK cells. Although many STAT proteins are involved in the IL-12 signalling pathway, STAT4 plays a significant role in inducing NK cell cytotoxicity and IFN- γ production via IL-12 (Wang et al., 2000). Thus, intracellular staining for p-STAT4 after stimulation of NK cells with IL-12 was performed (Fig 3.16 A). However, constitutive phosphorylation of STAT4 was observed for both stimulated and unstimulated PB and CB NK cells (Fig 3.16 B, C).

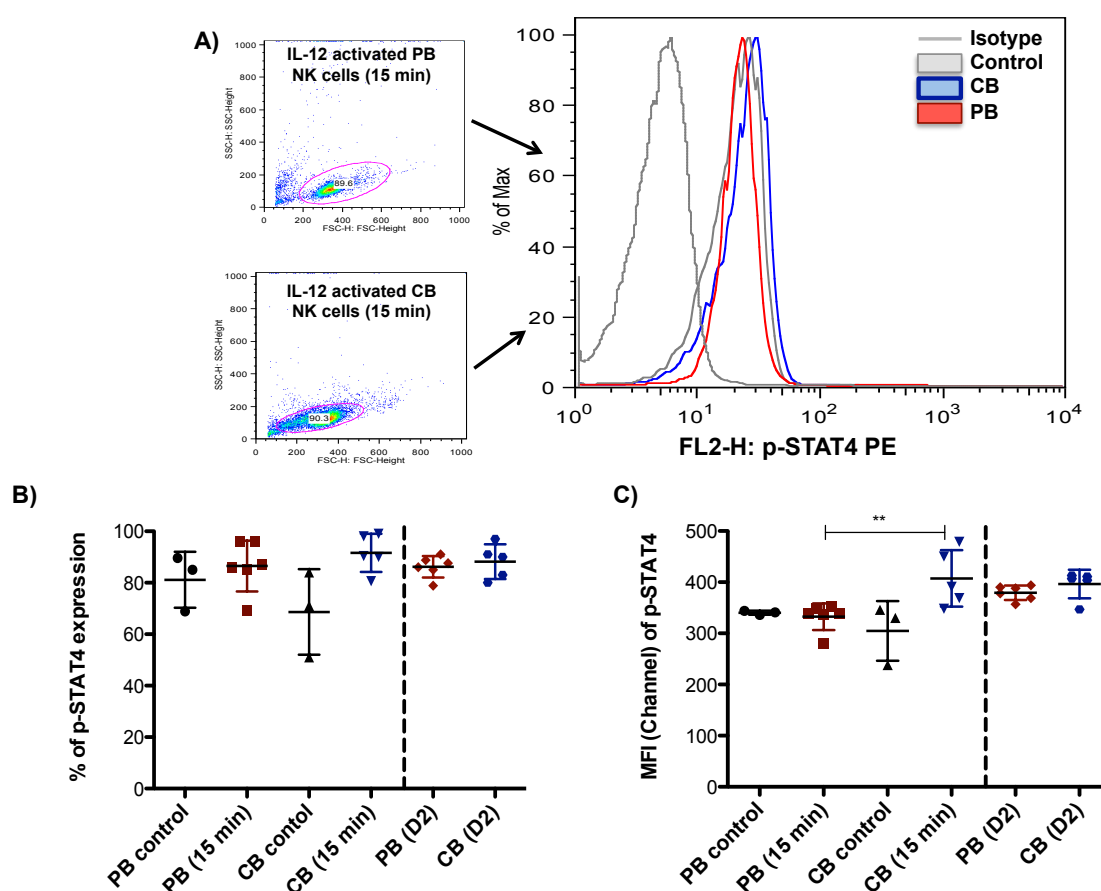


Figure 3.16 Analysis of phosphorylated STAT4 expression by cytokine-activated CB and PB NK cells. A) Flow cytometric analysis of the relative phosphorylation of STAT4 (p-STAT4) after 15 min stimulation of PB and CB NK cells with IL-12. B) Mean percentage of expression of p-STAT4 after 15 min stimulation with IL-12 C) Density (MFI) of p-STAT4 expression after stimulation with IL-12 for 15 min or 2 days for PB and CB NK cells. Analysis was performed on purified NK cells stimulated with IL-2 for 15 min or 2 days. Results are presented as Mean \pm SD. Control = unstimulated NK cells. PB N=5, CB N=5, Control N=3. $p < 0.01$ (**), and $p < 0.01$ (***).

3.2.6 Cytokine activation induces formation of an apoptotic population of PB and CB NK cells

As two populations of NK cells are noted on forward scatter *versus* side scatter flow cytometry analysis plots after stimulation with cytokine, NK cell viability and apoptotic state were assessed using 7AAD and Annexin V (Fig 3.17 A). Necrotic cells that have lost membrane integrity stain positive for 7AAD, whereas apoptotic cells that express phosphatidylserine (PS) stain positive for Annexin V. It was found that the smaller cells, with respect to forward scatter, were dead cells, as they were positive for 7AAD (Fig 3.17 A). In contrast, the cells that responded to cytokines were bigger (with respect to forward scatter) and represented live cells (Fig 3.17 A). Expression of the anti-apoptotic protein B-cell lymphoma (Bcl)-2 was then assessed for NK cells stimulated with cytokine using real-time PCR. Selective upregulation of Bcl-2 was observed for PB NK cells activated with IL-2 in comparison to CB NK cells activated with IL-2 ($p = 0.0298$) (Fig 3.17 B). In addition, Bcl-2 expression was not upregulated by PB or CB NK cells for any of the other cytokines tested which could explain the existence of an apoptotic population following cytokine activation, as seen in the viability study.

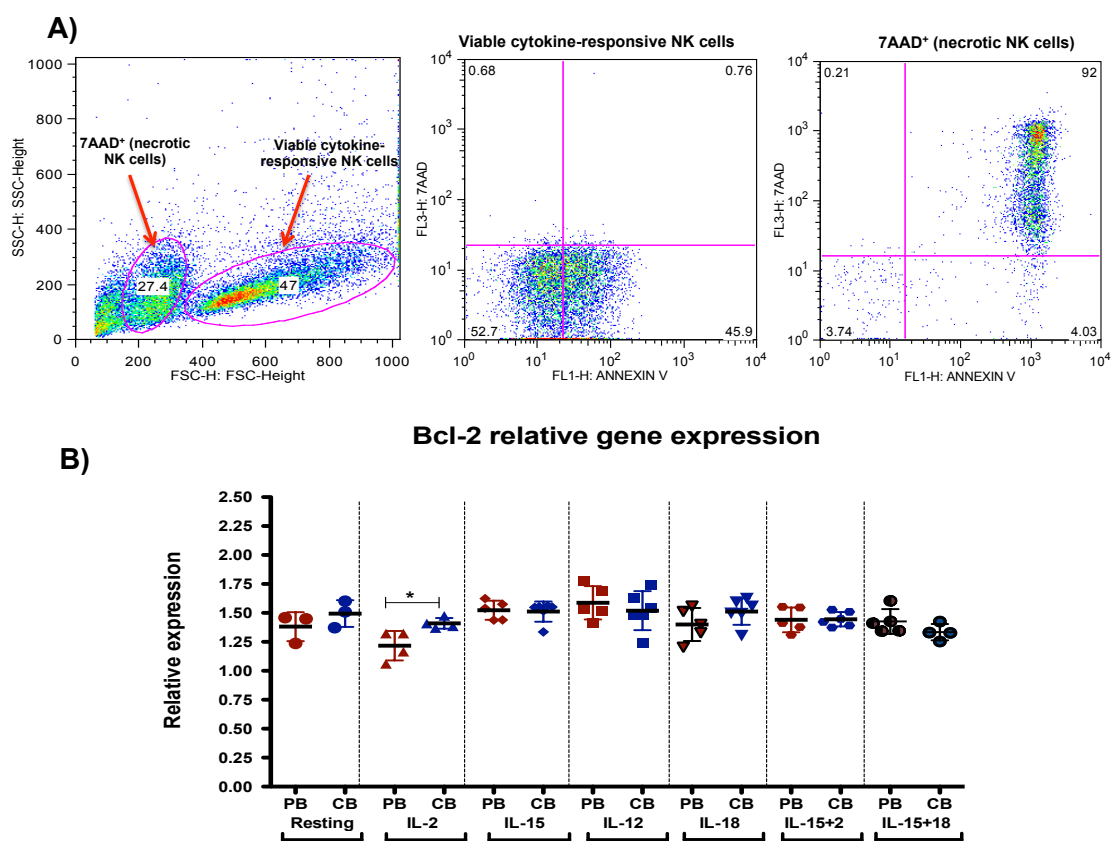


Figure 3.17 NK cell viability and expression of the anti-apoptotic marker Bcl-2 after stimulation of CB and PB NK cells with cytokines. A) Flow cytometry plot showing two populations gated based on forward-scatter *versus* side-scatter (left panel), Annexin V versus 7AAD staining gated on the viable cytokine-responsive NK cells (middle panel) or gated on the 7AAD⁺ necrotic NK cells (right panel). B) Relative Bcl-2 gene expression after 24 h stimulation with cytokines. mRNA quantity was normalised against three reference genes (ACTB, β 2M and UBC). Resting PB and CB NK cells N=3, cytokine-activated PB N=4 - 5, CB N=5 - 6. $p < 0.05$ (*).

3.3. Discussion

CB NK cells have unique phenotypic characteristics in comparison to PB NK cells. CB NK cells are reported to have higher NKG2A expression and low expression of KIR, adhesion molecules and some activating receptors (Dalle et al., 2005, Luevano et al., 2012a, Tanaka et al., 2003, Wang et al., 2007). These phenotypic features are indicative of CB NK cells being less mature, which may suggest that they are less functional. Therefore, the effects of cytokine activation on the phenotype of CB NK cells were analysed. A comprehensive comparison between PB NK cells and CB NK cells was performed to assess different mechanisms of activation and responsiveness to specific cytokine. The cytokines used to activate PB and CB NK cells were IL-2, IL-12, IL-15 and IL-18. Herein, the acquisition of an activated phenotype by CB and PB NK cells upon cytokine activation was confirmed.

CB NK cells were less activated following IL-2 stimulation than PB NK cells, as CB NK cells expressed lower levels of CD69 and NKp44 after exposure to IL-2. IL-2 binds to the α subunit of the IL-2 receptor with low affinity, whereas binding of IL-2 to the β chain and γ chain confers intermediate affinity. To achieve high affinity binding, association of the intermediate affinity chains (β and γ) with the α chain is required (Voss et al., 1992). Here, expression of the γ chain by NK cells was not investigated, as this molecule is undetectable at the surface of quiescent NK cells (David et al., 1998). In contrast, IL-2R β is expressed constitutively by resting PB and CB NK cells (Dalle et al., 2005, David et al., 1998, Luevano et al., 2012a) and is believed to play a significant role in initiating NK cell activation by IL-2 (Pillet et al., 2009, Pillet et al., 2008). Upon binding of IL-2 to IL-2R β , rapid expression of the γ chain occurs leading to increased responsiveness to IL-2. This is followed by the induction of IL-2R α expression, which is involved in high affinity binding, although this occurs with slower kinetics than for the induction of IL-2R γ expression. This may contribute to a sequential response of NK cells to IL-2 stimulation, which could be initiated by binding of IL-2 to the IL-2R β chain followed by phosphorylation of signalling components. Subsequently, this results in the expression of high-affinity receptors and upregulation of activation receptors leading to NK cell effector function (Pillet et al., 2009).

It has been reported that cytokine concentration is a key factor for activation of NK cells. High concentrations of cytokine may induce signalling through the intermediate-affinity chain (Nakamura et al., 1994, Waldmann, 2006). Although CD56^{bright} NK cells respond to picomolar doses of IL-2, CD56^{dim} NK cells require higher doses of IL-2 due to lower expression of IL-2R α (Cooper et al., 2001a). Thus, signalling events may be enhanced through the intermediate affinity receptors by increasing the dose of IL-2. Here, activation of CB NK cells required five times more IL-2 (1000 IU/mL) than PB NK cells (200 IU/mL), however the activation level observed was remarkably less for CB NK cells. Similar expression levels of IL-2R β by CB and PB CD56^{bright} and CD56^{dim} NK cells was found, but lower expression of IL-2R α by CB CD56^{dim} NK cells was observed, as compared to PB CD56^{dim} NK cells. Binding of IL-2 to IL-2R β is known to activate Janus Kinase (JAK) 1 which is downstream of STAT5, and phosphorylation of STAT5 induces the transcription of target genes. A significant reduction in p-STAT5 expression by CB NK cells was noted after stimulation with IL-2 as compared to PB NK cells. However, how this correlates with lower activation levels observed after stimulation with IL-2 is not known and needs further investigation. In addition, less phosphorylation of the STAT5 pathway downstream of the IL-2 receptors in CB NK cells may interfere with their responsiveness to IL-2 where STAT5 itself is a key regulator of IL-2R α gene expression (Lécine et al., 1996). Interestingly, I observed optimal activation of CB NK cells with IL-15, which also signals through STAT5. This implies that the reduced response of CB NK cells to IL-2 is a result of lower IL-2R α expression levels, rather than downstream signalling components.

A higher level of IL-15R α expression by CB CD56^{bright} NK cells than PB CD56^{bright} NK cells was observed. Unlike IL-2R α , IL-15R α provides high affinity binding to IL-15 and its cytoplasmic domain is directly connected to signalling components. The IL-15/IL-15R α complex also triggers signalling into IL-15-responsive neighbouring cells by a phenomenon called trans-presentation (Dubois et al., 2002, Huntington et al., 2009). This could explain why I observed maximal activation of CB NK cells using a low concentration of IL-15. Moreover, IL-15R α plays a critical role in NK cell development (Huntington et al., 2009). Although CB NK cells reach late stages of development, their maturation is incomplete. The IL-15/IL15R α -complex has been shown to be essential for the progression from a CD56^{bright}CD16⁻KIR⁻ phenotype to a CD56^{dim}CD16⁺KIR⁻ phenotype and finally to a mature CD56^{dim}CD16⁺KIR⁺ NK cell

phenotype (Huntington et al., 2009). Therefore, the higher expression of IL-15R α suggests an enhanced readiness of CB NK cells to respond to IL-15 exposure that may be needed to drive their final maturation.

Here, I showed that both subsets of CB NK cells express significantly less IL-12R β 1 but equivalent levels of IL-12R β 2 to PB NK cells. The IL-12R is composed of two low affinity chains; IL-12R β 1 and IL-12R β 2, in which cooperation of the β 1 and β 2 chains is required to initiate phosphorylation of IL-12 signalling components such as STAT4. Resting T cells broadly express IL-12R β 1, but scarcely express IL-12R β 2. In contrast, NK cells express IL-12R β 2 allowing them to respond promptly to IL-12 activation (Wang et al., 2000). Nonetheless, I observed enhanced responsiveness to IL-12 activation by CB NK cells. This suggests that IL-12R β 1 expression may be upregulated after stimulation with IL-12, although was not tested here. CB NK cells showed higher p-STAT4 MFI than PB NK cells after 15 min stimulation with IL-12, which may correspond with better activation. Yet, high background of STAT4 phosphorylation was observed for both stimulated and unstimulated NK cells, which could be a result of NK cell handling or processing.

A proportion of the cytokine-treated NK cells were apoptotic. This was observed in all cultures including PB and CB NK cells. It remains unknown whether these cells were exhausted after activation or die as a result of cytokine toxicity. However, it is more likely that these cells are not responsive to cytokine. Indeed, reduced expression of high affinity cytokine receptors by NK cells could lead to apoptosis due to lack of a survival signal. Bcl-2 is an anti-apoptotic protein and its upregulation by NK cells promotes their survival. While IL-2 promoted upregulation of Bcl-2 by PB NK cells, this was not observed for CB NK cells. IL-15 activation is known to upregulate Bcl-2 expression by NK cells to promote their survival (Carson et al., 1997). In this study, the cells were stimulated for 24 h because the quantity of the mRNA recovered was very low after longer incubation of NK cells with cytokines. This could be because the genes are transcribed and translated in order to assemble the Bcl-2 proteins, which yields insufficient mRNA to perform real-time PCR.

NK cell effector function can be triggered by a variety of receptors. These triggering receptors are defined as NCRs belonging to the immunoglobulin superfamily and are able to mediate NK cell cytotoxicity upon engagement of their ligands on target cells (Moretta et al., 2000). Among these receptors, NKp44 is exclusively expressed by activated NK cells and NKp44 upregulation by activated NK cells is involved in killing of tumour cells (Vitale et al., 1998). It has been shown that masking of NKp44 using monoclonal antibodies affects NK cell mediated killing of susceptible targets (Cantoni et al., 1999). NKp44 was upregulated by CB NK cells after activation with all cytokines used in this study but to a lesser extent with IL-2. The ligand for this receptor has recently been discovered (NKp44L), which is a variant of mixed-lineage leukaemia-5 (MLL5) (Baychelier et al., 2013). Elevated MLL5 levels were found to be associated with better outcomes in AML patients (Damm et al., 2011). Furthermore, NKp44L is expressed on a variety of haematopoietic and non-haematopoietic tumour cell lines and binds restrictively to NKp44 leading to lysis of tumour cells (Baychelier et al., 2013). High expression of NKp44, especially by NK cells activated with IL-15, was noted. Thus, IL-15 activated CB NK cells could potentially be effective against cancer cells in a NK cell-adoptive transfer therapy setting. Indeed, high levels of NKp44 expression by IL-15 activated CB NK cells is considered to be beneficial for NK cell-based therapy and IL-15 is currently being clinically tested in this context by Miller and colleagues (clinicaltrials.gov, trial number; NCT01385423). IL-15 is a better candidate for cytokine-based therapy as compared to other cytokines used in this study for several reasons. Firstly, IL-15 is required for NK cell homeostasis, expansion and survival *in vivo* (Carson et al., 1997, Evans et al., 1997). Secondly, NK cell development and differentiation is IL-15 dependent (Mrozek et al., 1996). Thus, IL-15 may enhance NK cell differentiation from CD34⁺ progenitors after HSCT promoting NK cell reconstitution and a GvL effect. Moreover, IL-15 does not promote the expansion of Treg cells, which was observed to hamper NK cell cytotoxicity in IL-2 based clinical trials (Barkholt et al., 2009, Geller et al., 2011).

NK cell activation is a cooperative process involving activating and inhibitory receptors. The strength of the transduced signal is largely dependent on the relative amounts of activating/inhibitory receptor expression (Wang et al., 2007). Inhibitory receptor expression by NK cells also correlates with their degree of maturity. NK cells at early stages of differentiation express high densities of CD94/NKG2A and scarce

amounts of CD16. The progression towards a fully mature NK cell is associated with decreased expression of CD94 and up-regulation of KIR. Hence, CD56^{dim}CD16^{bright} NK cells exhibit a more differentiated phenotype with high expression of KIR and enhanced killing capacity (Freud and Caligiuri, 2006). The results described here provide an indication that *in vitro* cytokine activation promotes activation of NK cells without changing the expression of their inhibitory receptors. In this regard, both resting and activated CB NK cells express higher CD94/NKG2A levels and lower levels of KIR indicating that they are less mature and have reduced cytotoxicity due to higher engagement of inhibitory receptors over activating ones. KIR mismatch is critical for inducing NK cell alloreactivity in HSCT. KIR2DL1⁺ NK cells are selectively able to lyse leukaemic blasts expressing C1 HLA group alleles whereas KIR2DL2/3⁺ NK cells are inhibited by C2 HLA group alleles (Pende et al., 2009). Yet, it is unknown how much KIR expression is needed to mediate a GvL effect. A study that aimed to predict how much KIR expression is required to mediate a GvL effect showed significant variation (0-62 %) in KIR expression for potential alloreactive NK cells based on both frequency and the type of missing KIR ligands (Fauriat et al., 2008). Herein, CB NK cells exhibited KIR expression, although to a lower extent than PB NK cells, suggesting that CB NK cells could still mediate alloreactivity. Indeed, the role of KIR mismatch in mediating CB NK cell alloreactivity after transplantation cannot be excluded (Beziat et al., 2009).

To summarise, cytokine activation induced an activated phenotype of CB NK cells. CB NK cells showed enhanced responsiveness to IL-15, IL-18 and IL-12, but were less responsive to IL-2 than PB NK cells. This was associated with lower expression of IL-2R α and reduced phosphorylation of STAT5 upon stimulation with IL-2 for CB NK cells. The data presented here shows that CB NK cells can respond to cytokine activation. Therefore, the functionality of CB and PB NK cells after cytokine stimulation was explored in the following chapters.

Chapter 4: Functional analysis of cytokine-activated NK cells

4.1 Introduction

During infection, activated DCs and macrophages release cytokines including IL-12, IL-15 and IL-18 leading to increased NK cell effector functions (Cooper et al., 2004). In addition, NK cell exposure to these cytokines during viral infection results in rapid proliferation and increased numbers of circulating NK cells (Biron et al., 1983). NK cell proliferation during the early phases of infection is non-specific (Dokun et al., 2001, Yokoyama et al., 2004), however, specific proliferation of a subset of NK cells that preferentially express Ly49H receptors was observed in a MCMV mouse model at late phases of infection (Dokun et al., 2001, French et al., 2006b). The influence of cytokines on proliferation of PB NK cells has been intensively investigated. Typically, cytokines of the common γ chain family, IL-2 and IL-15, appear to be key for promoting NK cell expansion (Ma et al., 2006). In addition, synergistic proliferative responses were seen following stimulation of NK cells with a combination of IL-2 and IL-12 (Wang et al., 2000), IL-2 and IL-18 (Son et al., 2001), or a combination of IL-15 and IL-18 (French et al., 2006a).

As for CB NK cells, it has been shown that IL-2 stimulation induces expansion of CB NK cells (Dalle et al., 2005, Wei et al., 2005). Dalle *et al.* showed that CB NK cells respond to nanomolar doses of IL-2 exhibiting proliferative capacity similar to PB NK cells after 7 days of stimulation (Dalle et al., 2005). Conversely, it was shown by our group that although CB NK cells divide after IL-2 stimulation, their proliferative response is slower than PB NK cells (Luevano et al., 2012a). In addition, it was reported that CB NK cells require higher doses of IL-2 to expand *in vitro* (Condiotti et al., 2001, Luevano et al., 2012a). However, proliferation of CB NK cells in response to other key cytokines requires further study.

In contrast to PB NK cells, resting CB NK cells are unable to lyse K562 cells with high efficiency (Dalle et al., 2005, Gaddy et al., 1995, Luevano et al., 2012a). However, the cytotoxic function of CB NK cells could be enhanced upon stimulation with IL-2 (Condiotti et al., 2001, Gaddy et al., 1995, Luevano et al., 2012a, Xing et al., 2010), IL-12 (Gaddy et al., 1995), IL-15 (Gaddy and Broxmeyer, 1997) or IL-18 (Nomura et al.,

2001). Killing of K562 cells by NK cells occurs via the granzyme/perforin-mediated pathway whereby these molecules induce damage to the target cell membrane and cell death (Vujanovic et al., 1996). Notably, poor killing capacity by resting CB NK cells seems to be associated with low expression of the cytotoxic mediators granzyme B and perforin (Luevano et al., 2012a, Wang et al., 2007). Yet, the effect of other cytokines on granzyme B and perforin expression and cytotoxicity of CB NK cells is not known.

Upon stimulation, NK cells release IFN- γ and TNF- α that act as a co-stimulatory signal for other immune cells (Cooper et al., 2004), inhibit tumour growth (Dighe et al., 1994, Ikeda et al., 2002) and enhance killing through upregulation of specific receptors on target cells (Wang et al., 2012). Reports analysing IFN- γ production by CB NK cells show inconsistent results. Comparable production of IFN- γ by CB and PB NK cells after PMA/ionomycin stimulation was described by Dalle *et al.* and Wang *et al.* (Dalle et al., 2005, Wang et al., 2007). Conversely, it was shown by Luevano *et al.* that although CB CD56^{bright} NK cells have comparable intracellular IFN- γ levels to PB CD56^{bright} NK cells, CB CD56^{dim} NK cells express significantly less intracellular IFN- γ than PB CD56^{dim} NK cells (Luevano et al., 2012a). Similarly, CB NK cells express less intracellular TNF- α than PB NK cells (Krampera et al., 2000). Cytokine stimulation, in particular with IL-12 and IL-18, induces the production of IFN- γ and TNF- α by PB NK cells (Fehniger et al., 1999). However, the repertoire of cytokines elaborated by CB NK cells upon cytokine activation remains to be investigated as most of the existing studies analysed intracellular cytokine expression, which does not necessarily correlate with their secretion.

To our knowledge, many features of CB NK cell functionality upon cytokine activation have not been investigated. Proliferation of CB NK cells was shown after IL-2 stimulation but it is still unknown whether other cytokines can induce better proliferation of CB NK cells. Furthermore, the killing capacity of CB NK cells was investigated following stimulation with cytokines in separate studies but they did not address whether these cytokines impact on granzyme B and perforin expression at the transcriptional level.

In this chapter, I explored the effect of cytokine activation on CB NK cell proliferation and compared it to PB NK cells. Furthermore, I analysed mRNA levels of granzyme B

and perforin in PB and CB NK cells following stimulation with cytokines and correlated gene expression with cytotoxicity. In addition, secretion of cytokines by cytokine-activated CB NK cells was compared to PB NK cells using multiplex assays (bead-based immunoassay) in order to identify the specific cytokine secretion patterns of different sources of NK cells. The response of PB and CB NK cells to different cytokine conditioning regimes will help determine whether different mechanisms of activation exist between these two NK cell sources.

4.2 Results

4.2.1 Assessment of NK cell proliferation after cytokine stimulation

4.2.1.1 PB and CB NK cells showed the greatest tendency to proliferate in response to IL-15+IL-18

The majority of NK cells are quiescent *in vivo* and their expansion occurs following exposure to cytokines such as IL-12 and IL-15 produced by activated monocytes, or IL-2 produced by activated T cells (Warren, 1996). Herein, proliferation of NK cells was tracked using CFSE after cytokine stimulation (Table 2.12). CFSE-labelled NK cells from both CB and PB showed a similar pattern of proliferation at day 2 regardless of the cytokine used (Fig 4.1 and Fig 4.2 respectively). Therefore, it is possible that reduced CFSE fluorescence at day 2 resulted from a decay of CFSE intensity rather than actual cellular divisions, however this could not be tested as unstimulated CB NK cells will undergo apoptosis in this timeframe. At day 5, however, PB and CB NK cells showed differential proliferation in response to cytokines. PB NK cells showed the highest tendency to proliferate in response to IL-15+IL-18 (Fig 4.1 D), followed by IL-2 (Fig 4.1 A), IL-15+IL-2 (Fig 4.1 C), and IL-15 (Fig 4.1 B). Different proliferation responses to cytokines were seen for CB NK cells, with IL-15+IL-18 (Fig 4.2 D) showing the greatest proliferative response, followed by IL-15+IL-2 (Fig 4.2 C) and IL-15 (Fig 4.2 B). Notably, CB NK cells activated with IL-2 did not proliferate further between days 2 and 5 (Fig 4.2 A). Altogether, activation with IL-15+IL-18 induced optimum expansion of PB and CB NK cells post-cytokine stimulation. Figure 4.3 shows the proliferation from 3 independent experiments performed on PB and CB NK cells.

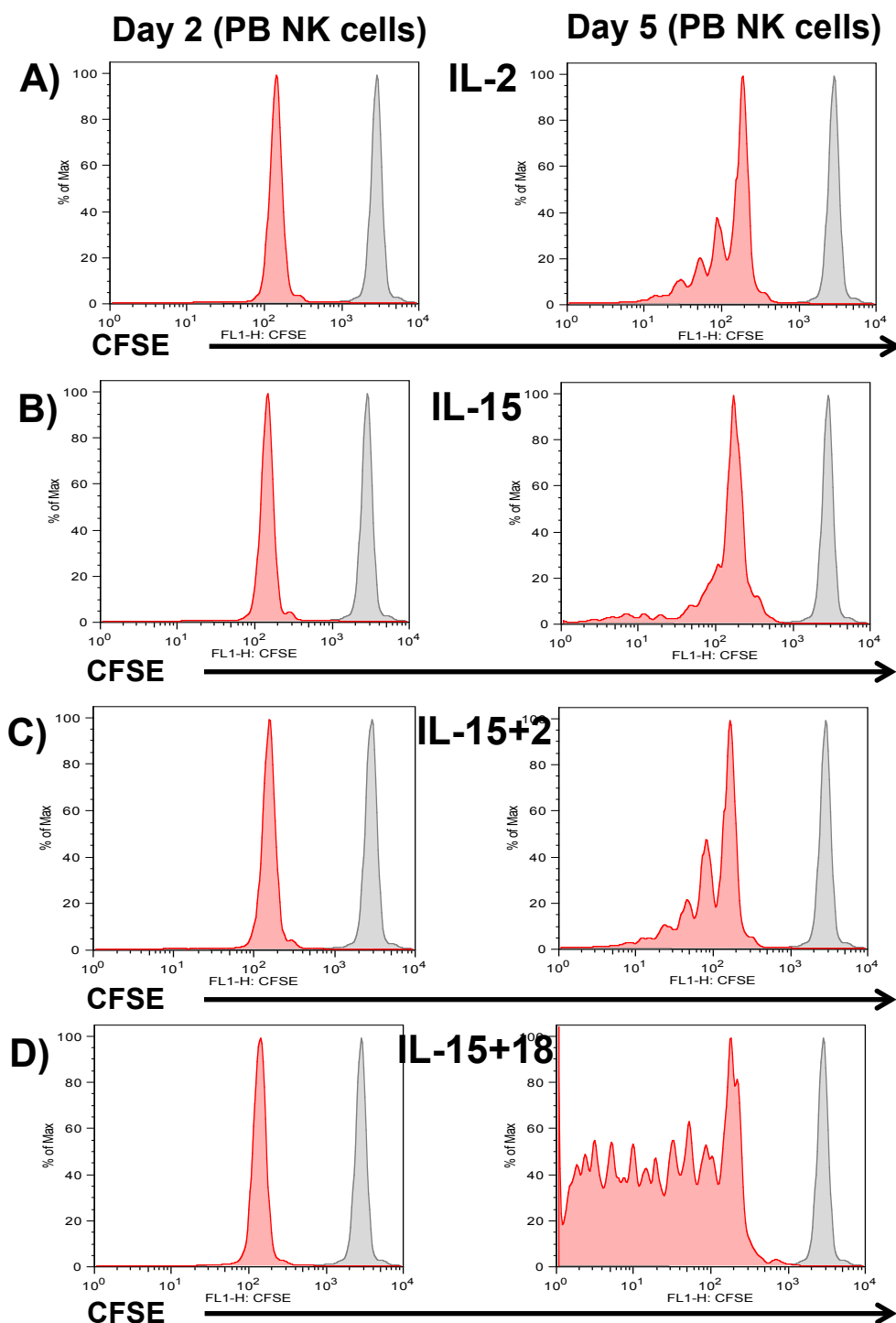


Figure 4.1 Proliferation of PB NK cells in response to stimulation with cytokines. CFSE dilution by PB NK cells activated with IL-2 (A), IL-15 (B), IL-15+IL-2 (C) and IL-15+IL-18 (D) assessed at day 2 (left panel) and day 5 (right panel) post-stimulation with cytokines. Grey peak indicates CFSE staining at day 0. Histograms show representative data from four independent experiments.

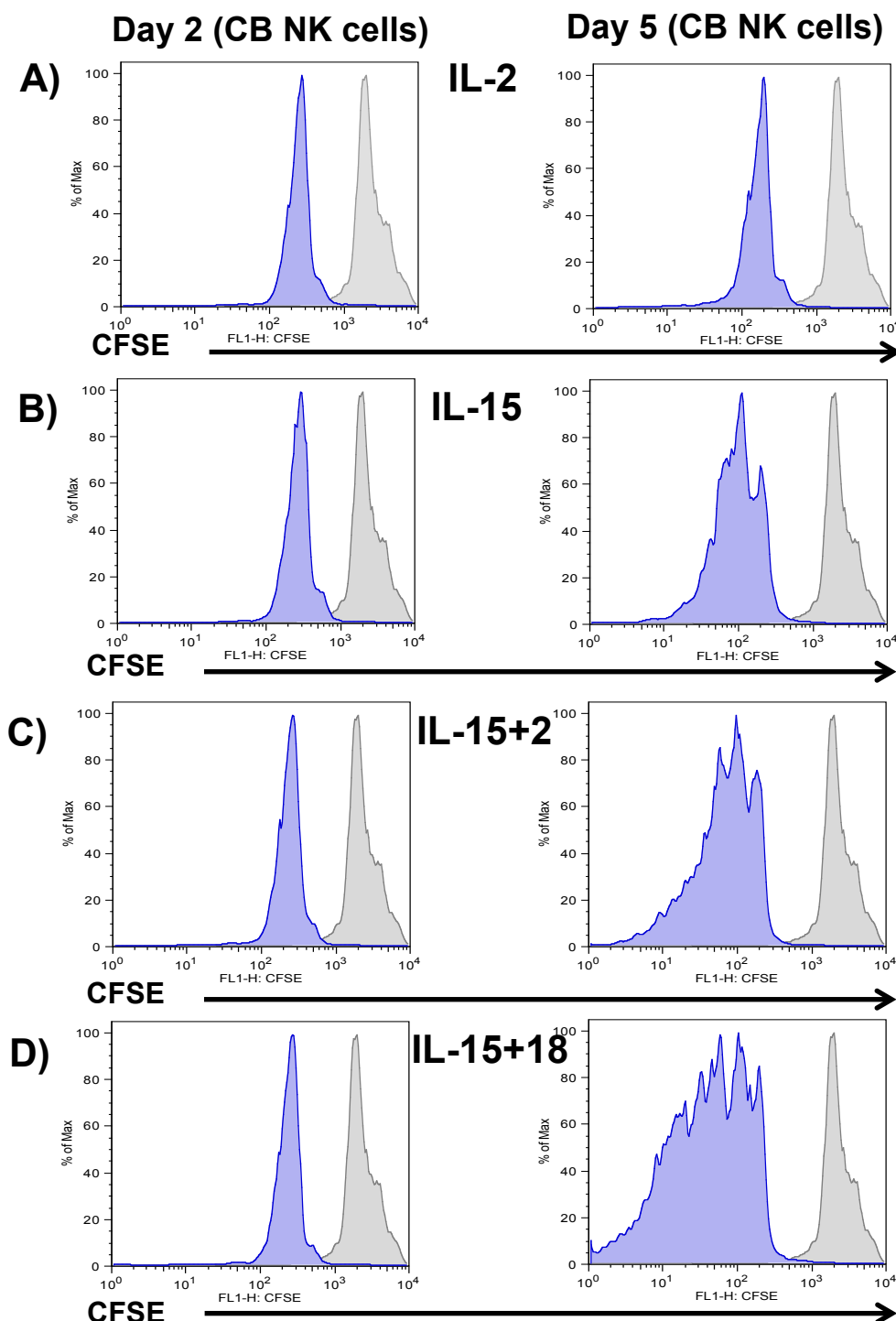


Figure 4.2 Proliferation of CB NK cells in response to stimulation with cytokines. CFSE dilution by CB NK cells activated with IL-2 (A), IL-15 (B), IL-15+IL-2 (C) and IL-15+IL-18 (D) assessed at day 2 (left panel) and day 5 (right panel) post-stimulation with cytokines. Grey peak indicates CFSE staining at day 0. Histograms show representative data from four independent experiments.

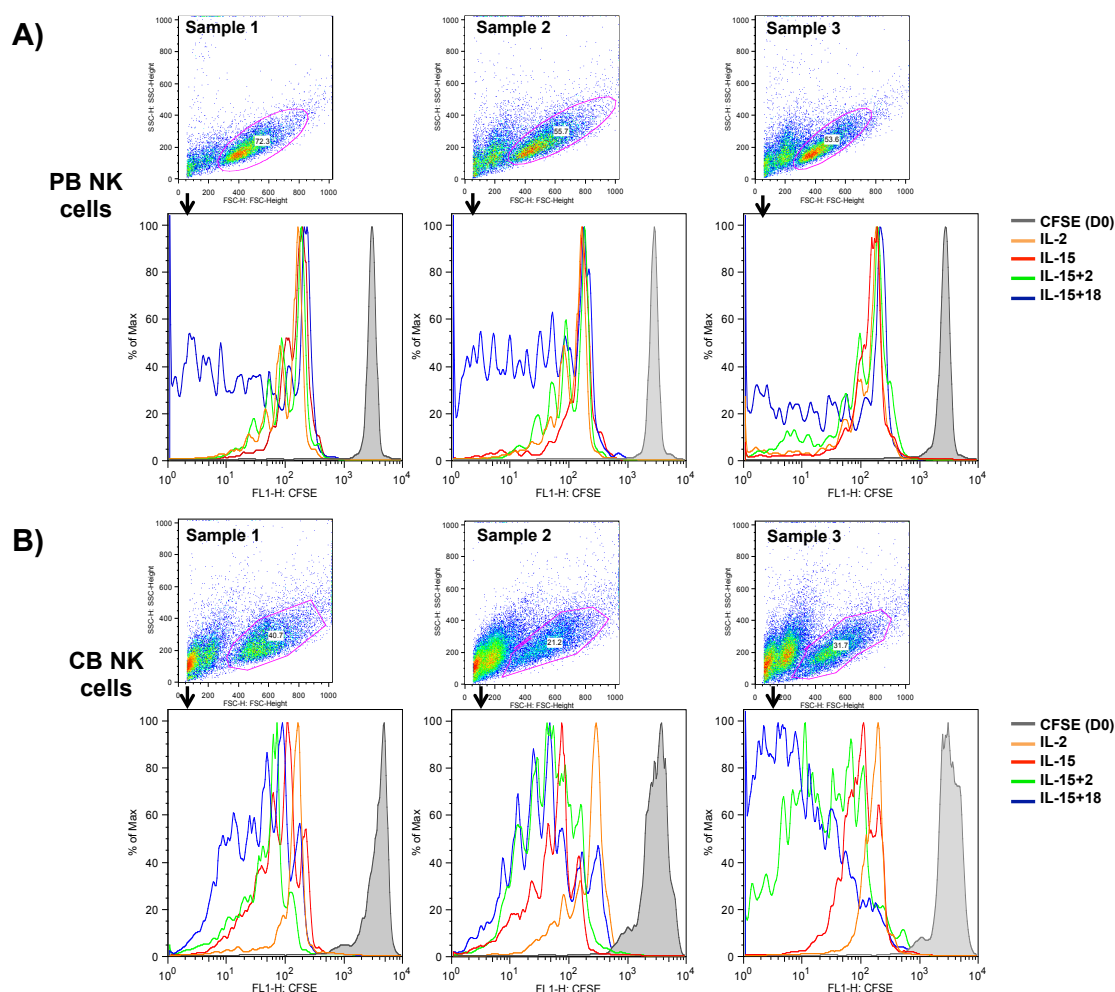


Figure 4.3 NK cell proliferation after 5 days stimulation with cytokine. A) CFSE dilution by 3 samples of PB NK cells stimulated with IL-2, IL-15, IL-15+IL-2 and IL-15+18 for 5 days. B) CFSE dilution by 3 samples of CB NK cells stimulated with IL-2, IL-15, IL-15+IL-2 and IL-15+18 for 5 days.

4.2.2 Assessment of NK cell cytotoxicity after cytokine stimulation

Although resting PB NK cells exhibit cytotoxicity against K562 cells, their cytolytic function is enhanced upon stimulation with cytokines. In contrast to PB NK cells, resting CB NK cells showed poor cytotoxicity in previous studies and their cytolytic function was only induced after cytokine stimulation (Dalle et al., 2005, Gaddy et al., 1995, Luevano et al., 2012a). In order to identify which cytokine or combination of cytokines promotes optimum cytolytic function of NK cells, a classical 4 h-⁵¹Cr-release assay was performed. K562 cells were used as target cells at different effector to target ratios including 1:1, 5:1 and 10:1. Effector NK cells were stimulated with cytokines prior to co-culture with K562 cells.

4.2.2.1 PB NK cells demonstrated enhanced killing capacity post-stimulation with IL-2, IL-15 or IL-12, whereas optimal killing capacity of CB NK cells was observed post-stimulation with IL-15 or with the combination of IL-15+IL-2

Cytotoxicity of cytokine-activated NK cells against K562 cells was assessed using a ^{51}Cr -release assay. Figure 4.4 shows a comparison between the killing of K562 cells by PB and CB NK cells at day 2 of stimulation with different cytokines. Interestingly, IL-2 activated CB NK cells showed poor killing capacity at day 2 post IL-2 stimulation at E:T ratios of 5:1 (Fig 4.4 B) and 10:1 (Fig 4.4 C) (% of specific lysis = 14.26 ± 2.87 % and 22.36 ± 4.86 % respectively) as compared to PB NK cells at the same E:T ratios (26.28 ± 1.61 % and 40.67 ± 4.85 %, $p = 0.0108$ and $p = 0.0372$ respectively). However, the cytotoxicity of CB NK cells had increased at day 5 post-stimulation and was comparable to PB NK cells (Fig 4.5). For IL-2 activated CB NK cells, an E:T ratio of 1:1 was not sufficient to induce killing of K562 cells, whereas better killing was seen by PB NK cells at this ratio (Fig 4.4 A, 4.5 A).

NK cell killing capacity was substantially enhanced by IL-15 as compared to IL-2 for both CB and PB NK cells at day 2 (Fig 4.4). This was shown at E:T ratios of 10:1 (PB = 51.07 ± 4.16 % and CB = 45.28 ± 8.10 %) (Fig 4.4 A) and 5:1 (PB = 42.32 ± 4.54 % and CB = 35.93 ± 8.80 %) (Fig 4.4 B). However, the cytolytic activity of CB NK cells was reduced by day 5 post-stimulation at E:T ratios of 5:1 and 10:1 (23.53 ± 10.40 % and 30.09 ± 8.96 % respectively) (Fig 4.5 B and C).

Stimulation of PB NK cells with IL-12 promoted significantly higher killing of K562 cells than IL-12 stimulated CB NK cells (Fig 4.4). Interestingly, lower ratios of effector cells (E:T ratios of 1:1 or 5:1) were required for killing by IL-12 activated PB NK cells (Fig 4.4 A, B). Percent specific lysis of K562 cells by IL-12 activated PB NK cells (5:1 E:T ratio) was 46.56 ± 5.67 %, whereas specific lysis was significantly less for CB NK cells (22.13 ± 6.89 %) ($P = 0.0258$). Similarly, greater killing was seen for PB NK cells at a 10:1 E:T ratio when compared to CB NK cells (50.53 ± 7.91 % and 31.96 ± 8.169 % respectively).

Although IL-18 promoted killing of K562 cells by PB NK cells, it did not induce similar activity by CB NK cells (Fig 4.4). This was found at all E:T ratios used in this study. At a 1:1 ratio, specific lysis was 8.31 ± 1.78 % by PB and 1.60 ± 1.12 % by CB

NK cells, $p = 0.023$ (Fig 4.4 A). Increasing the number of effector cells to a 5:1 ratio also had no effect on the killing by CB NK cells (6.26 ± 2.480 %) as compared to PB NK cells (29.91 ± 3.20 %) ($p = 0.0007$) (Fig 4.4 B). Similarly, CB NK cells showed lower cytotoxicity at a ratio of 10:1 (11.32 ± 4.82 %) compared to PB NK cells (34.88 ± 3.14 %), $p = 0.0026$ (Fig 4.4 C).

Comparable killing capacity for both IL-15+IL-2 activated PB and CB NK cells was seen at day 2 (Fig 4.4). However, CB NK cells showed significantly higher killing capacity at day 5 (Fig 4.5). At a 10:1 E:T ratio, mean percent specific lysis by CB NK cells was 46.69 ± 4.80 %, whereas for PB NK cells it was 31.54 ± 2.75 % ($p = 0.0338$) (Fig 4.5 C). The killing capacity of IL-15+IL-18 activated CB NK cells was significantly lower than IL-15+IL-18 activated PB NK cells at day 2 for all of the ratios examined including a 1:1 ratio, (Fig 4.4 A), 5:1 ratio (Fig 4.4 B) and 10:1 ratio (Fig 4.4 C) ($p = 0.0025$, $p = 0.0023$ and $p = 0.0003$ respectively). However, IL-15+IL-18 activated CB NK cells exhibited improved killing capacity after 5 days of cytokine stimulation (Fig 4.5). At an E:T ratio of 10:1, CB NK cells showed a specific lysis as high as 36.93 ± 1.79 %, which was greater than the specific lysis measured for PB NK cells at the same time point (28.71 ± 4.258 %) (Fig 4.5 C). Overall, CB NK cells showed maximal cytotoxicity following IL-15+IL-2 stimulation, whereas PB NK cells showed the highest cytotoxicity after IL-15 and IL-12 stimulation.

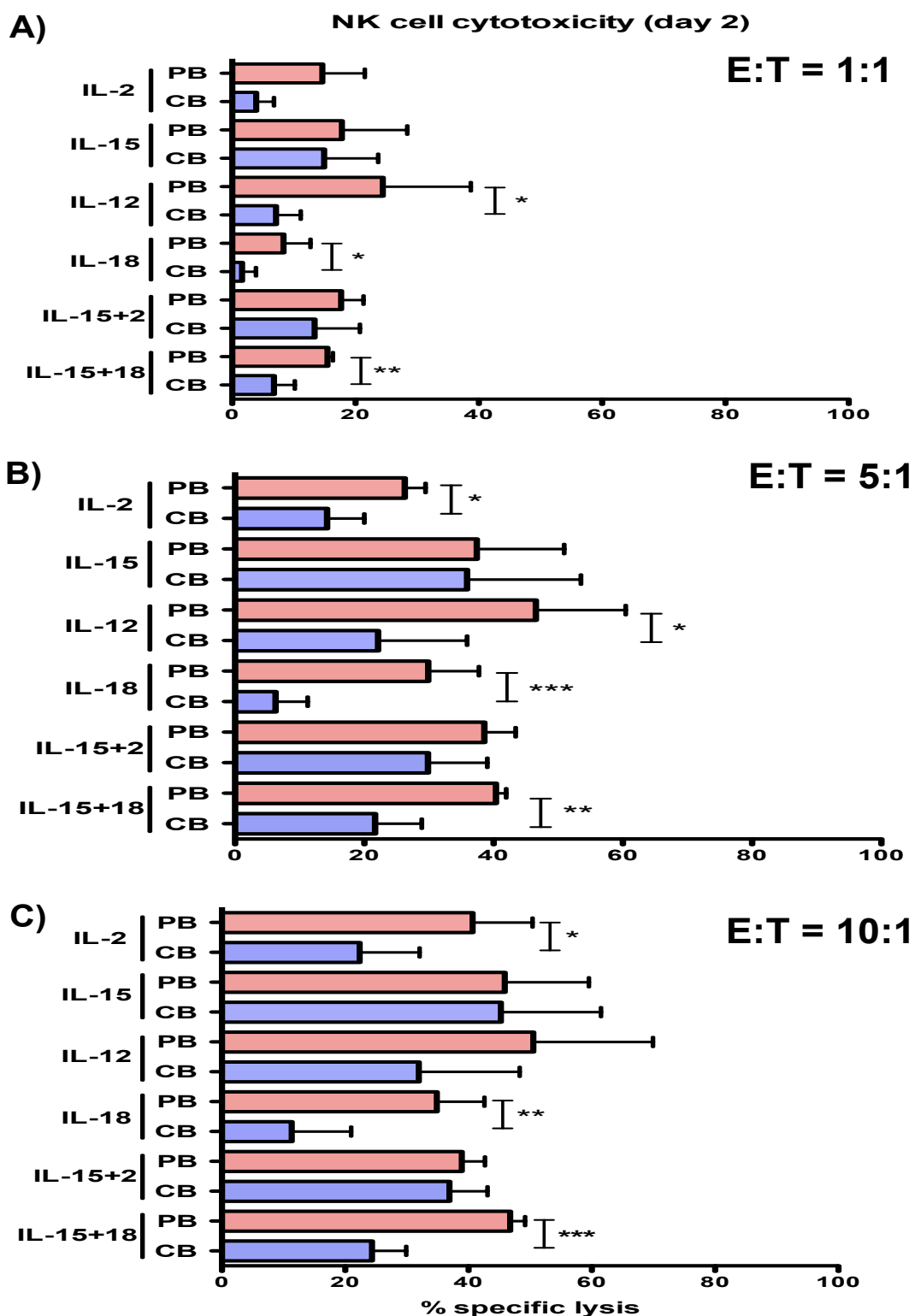


Figure 4.4 Killing of K562 cells by cytokine-activated NK cells 2 days after stimulation with cytokines. Comparison of PB (red) and CB (blue) NK cell cytotoxicity against K562 cells examined at E:T ratios of 1:1 (A), 5:1 (B) and 10:1 (C) by ^{51}Cr -release assay. E:T = effector to target ratio. N=4. p (*) < 0.05, p < 0.01 (**), and p < 0.001 (***). Specific lysis was calculated as (experimental release – spontaneous release) ÷ (maximum release – spontaneous release) × 100. Results are presented as Mean ± SD.

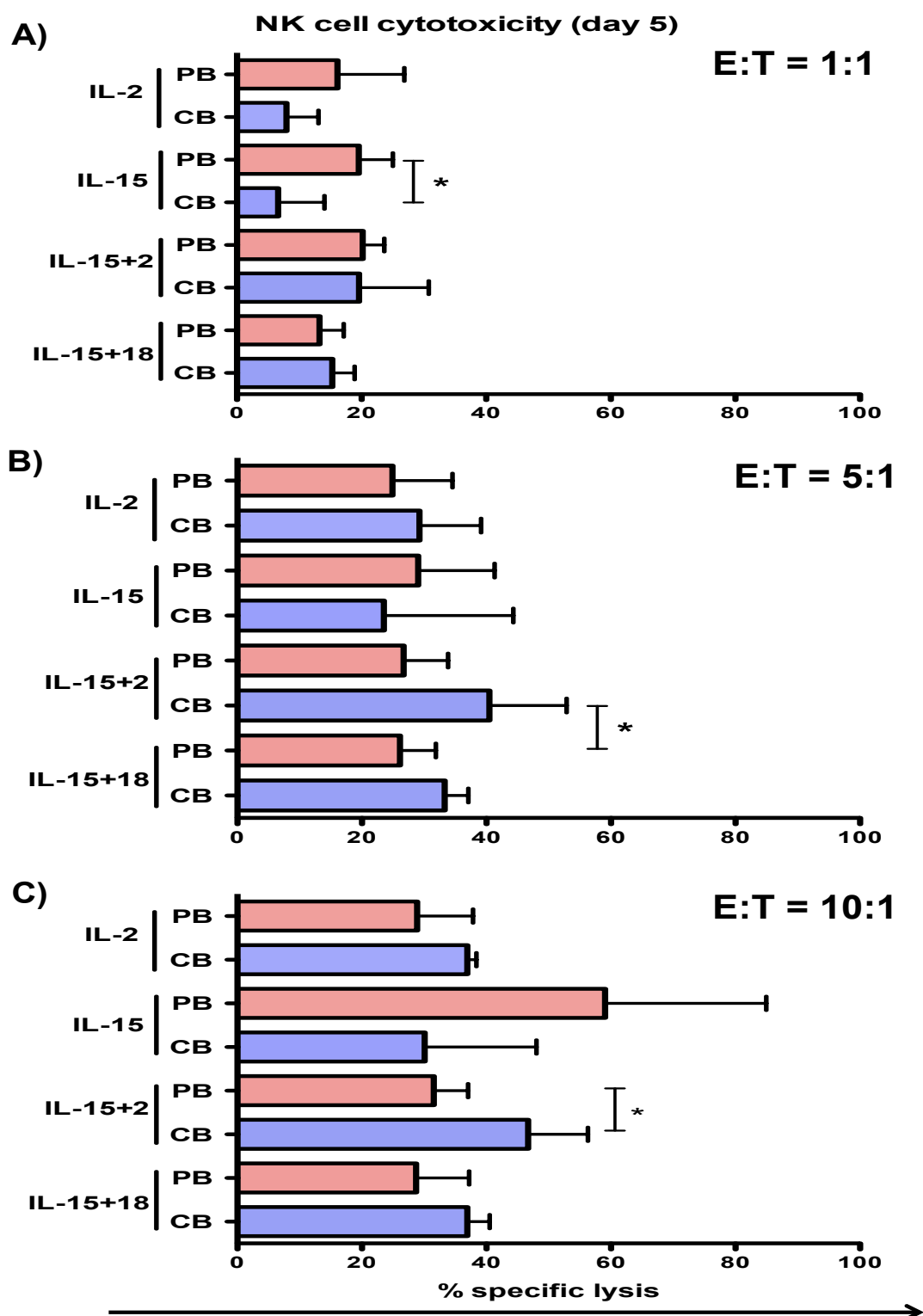


Figure 4.5 Killing of K562 cells by cytokine-activated NK cells 5 days after stimulation with cytokines. Comparison of PB (red) and CB (blue) NK cell cytotoxicity against K562 cells examined at E:T ratios of 1:1 (A), 5:1 (B) and 10:1 (C) by ^{51}Cr -release assay. E:T = effector to target ratio. N=4. p (*) < 0.05, (*), p <0.01 (**), and p <0.001 (***). Specific lysis was calculated as (experimental release – spontaneous release) ÷ (maximum release – spontaneous release) × 100. Results are presented as Mean ± SD.

4.2.2.2 Cytokine activation induced comparable granzyme B gene expression by PB and CB NK cells except for CB NK cells activated with IL-18 or the combination of IL-15+IL-18 that showed significantly less granzyme B expression than PB NK cells

To determine whether the expression of granzyme B and perforin correlates with NK cell cytotoxicity, the relative granzyme B and perforin gene expression was assessed using real-time PCR. IL-2 activated PB NK cells had higher relative gene expression of granzyme B than IL-2 activated CB NK cells, but the difference was not significant (Fig 4.6 A). In contrast, comparable levels of perforin were detected in both resting and IL-2 activated PB and CB NK cells (Fig 4.6 B). No distinctive granzyme B (Fig 4.6 A) or perforin (Fig 4.6 B) gene expression was found for either resting or for IL-12 or IL-15 activated CB NK cells or PB NK cells. Interestingly, IL-18 activated CB NK cells showed significantly less granzyme B (Fig 4.6 A) and perforin (Fig 4.6 B) expression as compared to PB NK cells, $p = 0.0407$. These results indicate that resting CB NK cells have higher granzyme B expression than those activated with IL-18, $p = 0.0367$.

Resting and IL-15+IL-2 activated PB and CB NK cells showed equivalent granzyme B (Fig 4.6 A) and perforin (Fig 4.6 B) gene expression. In contrast, activation with IL-15+IL-18 induced different granzyme B gene expression in PB and CB NK cells. Interestingly, granzyme B gene expression was significantly lower for CB NK cells activated with IL-15+IL-18 as compared to PB NK cells, $p = 0.0005$. This could explain the lower killing capacity observed for CB NK cells at day 2 post-stimulation with IL-15+IL-18. Moreover, granzyme B gene expression was significantly upregulated in IL-15+IL-18 activated PB NK cells as compared to resting PB NK cells, $p = 0.0033$ (Fig 4.6 A). Conversely, expression of perforin was not affected by IL-15+IL-18 stimulation for either PB or CB NK cells (Fig 4.6 B). Altogether, a trend toward higher granzyme B expression in PB NK cells than CB NK cells although not significant, whereas perforin levels appeared to be comparable between both sources and not impacted by cytokine stimulation.

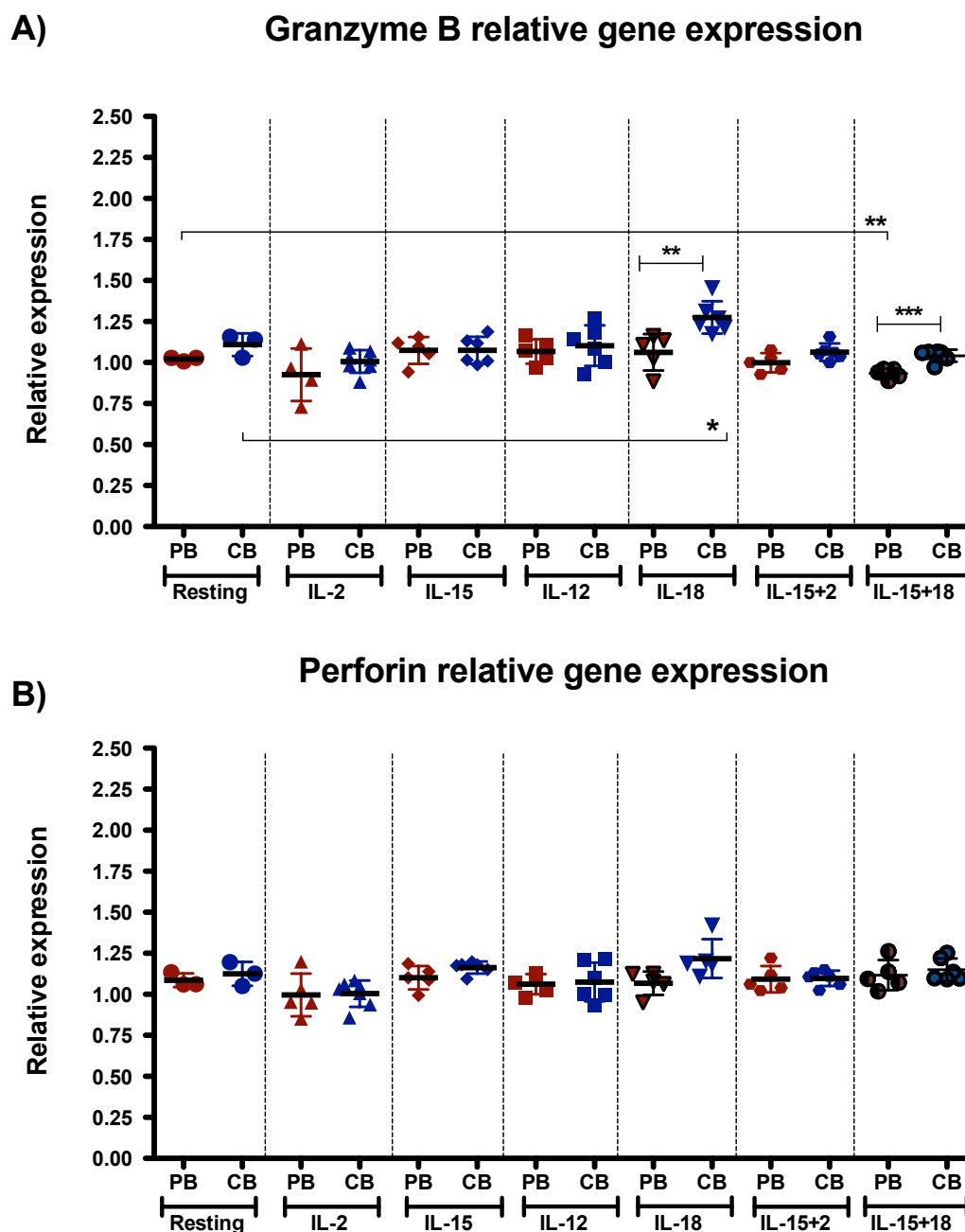


Figure 4.6 Analysis of granzyme B and perforin gene expression by cytokine-activated NK cells. Real-time PCR analysis of granzyme B (A) and perforin (B) relative gene expression by cytokine-activated NK cells, as compared to resting NK cells. mRNA quantity was normalised against three reference genes (ACTB, β 2M and UBC). Results are presented as Mean \pm SD. Resting NK cells (N=3), cytokine-activated NK cells from PB (N=5) and from CB (N=6).

4.2.3 Cytokine secretion by cytokine-activated NK cells

Secretion of cytokines into culture supernatants was analysed using multiplex (bead-based immunoassays) at days 2 and 5 post-stimulation with cytokines. Secretion of IFN- γ , IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, TNF- α , and TNF- β by cytokine-activated NK cells was analysed.

4.2.3.1 PB NK cells secreted the highest concentrations of IFN- γ post-stimulation with IL-2, IL-15 or IL-15+IL-18, whereas CB NK cells showed substantial secretion of IFN- γ upon stimulation with IL-12 or IL-15+IL-18

Significantly higher secretion of IFN- γ by PB NK cells was observed as compared to CB NK cells at days 2 and 5 post-stimulation with IL-2 (10411 ± 846.0 pg/mL and 1917 ± 1009 pg/mL, $p = 0.0002$) (Fig 4.7 A). Similarly, PB NK cells secreted substantial amounts of IFN- γ at day 5 (10130 ± 3034 pg/mL) compared to CB NK cells (1380 ± 615 , $p = 0.0301$) (Fig 4.7 B). This was associated with significant upregulation of IFN- γ gene expression by PB NK cells activated with IL-2 compared to resting PB NK cells ($p = 0.0063$) (Fig 4.7 C).

Both PB and CB NK cells secreted high amounts of IFN- γ upon stimulation with IL-15 (Fig 4.7). Upon analysing IFN- γ gene expression by real-time PCR, it was revealed that PB NK cells had significantly higher IFN- γ than CB NK cells after stimulation with IL-15, $p = 0.0145$ (Fig 4.7 C). Furthermore, a significant difference in IFN- γ gene expression was observed when IL-15 activated PB NK cells were compared to resting PB NK cells, $p = 0.0285$ (Fig 4.7 C).

Interestingly, IL-12 activated CB NK cells secreted high amounts of IFN- γ (12853 ± 4820 pg/mL) that significantly exceeded the amount secreted by PB NK cells (3757 ± 1268 pg/mL) ($p = 0.0441$) (Fig 4.7 A). Correspondingly, IL-12 activated CB NK cells showed significantly higher IFN- γ gene expression as compared to IL-12 activated PB NK cells, $p = 0.0051$ (Fig 4.7 C). Furthermore, IFN- γ mRNA was found to be significantly increased in IL-12 activated CB NK cells, as compared to resting CB NK cells, $p = 0.0114$ (Fig 4.7 C). Stimulation with IL-18 resulted in higher IFN- γ secretion by PB NK cells (5545 ± 2104 pg/mL) than CB NK cells (2678 ± 1765 pg/mL) (Fig 4.7

A). In accordance, IL-18 activation promoted higher IFN- γ gene expression in PB NK cells than CB NK cells (Fig 4.7 C), $p = 0.0077$.

Stimulation with IL-15+IL-2 led to high levels of IFN- γ secretion from both PB and CB NK cells (Fig 4.7 A, B). Moreover, real-time PCR analysis indicated that IFN- γ gene expression was significantly increased in cytokine-activated PB NK cells, as compared to resting PB NK cells, $p = 0.0022$ (Fig 4.7 C). Similarly, IL-15+IL-2 activated CB NK cells showed a slight increase in IFN- γ mRNA levels compared to resting CB NK cells (Fig 4.7 C).

High secretion of IFN- γ by both IL-15+IL-18 activated PB and CB NK cells was observed at both days 2 and day 5 post-stimulation (Fig 4.7 A, B). However, CB NK cells showed selective responsiveness to this combination, as the secretion was drastically higher at day 2 (20875 ± 2942 pg/mL) and at day 5 (15949 ± 2628 pg/mL) in comparison to PB NK cells, ($p = 0.0096$ and 0.0422) respectively. Interestingly, this was associated with a significant increase in IFN- γ gene expression by IL-15+IL-18 activated PB ($p = 0.0004$) and CB NK cells ($p = 0.0340$), as compared to resting NK cells (Fig 4.7 C). Overall, IL-12 or the combination of IL-15+IL-18 induced the highest secretion of IFN- γ by CB NK cells that was associated with upregulation of IFN- γ mRNA levels, whereas PB NK cells secreted high levels of IFN- γ post-stimulation with IL-2, IL-15 or IL-15+IL-18.

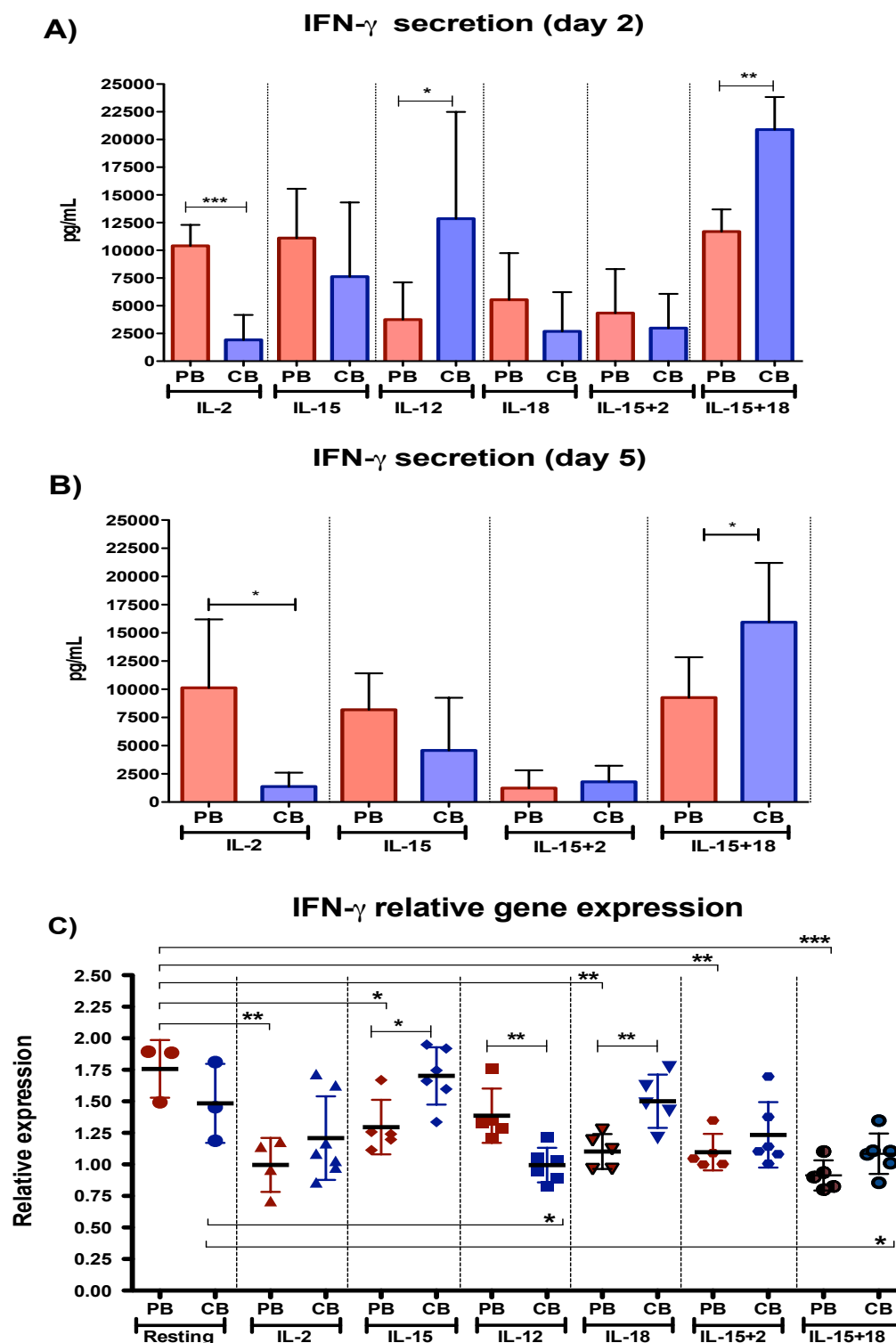


Figure 4.7 Analysis of IFN- γ secretion and gene expression by cytokine-activated NK cells. A) Assessment of IFN- γ secretion by cytokine-activated PB (red) and CB (blue) NK cells tested using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. C) Relative gene expression of IFN- γ analysed by real-time PCR for cytokine-activated PB and CB NK cells compared to resting NK cells. mRNA quantity was normalised against three reference genes (ACTB, β 2M and UBC). Results are presented as Mean \pm SD. Resting NK cells N=3, PB N=5 and CB N=6. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

4.2.3.2 IL-18 or IL-15+IL-18 activated CB NK cells secreted higher amounts of TNF- α than PB NK cells at day 2 post-stimulation

Differential secretion of TNF- α was observed following stimulation with different cytokines (Fig 4.8). CB NK cells activated with IL-18 secreted significantly higher amounts of TNF- α than PB NK cells activated with the same cytokine (Fig 4.8 A, $p = 0.0114$). Similarly, higher concentrations were secreted by CB NK cells activated with IL-15+IL-18 (331.9 ± 78.96 pg/mL) than PB NK cells (92.94 ± 14.96 pg/mL, $p = 0.0178$). Moreover, equivalent levels of TNF- α secretion by IL-15 activated PB NK cells were observed at day 2 (Fig 4.8 A). Notably, TNF- α levels decreased by day 5 of stimulation, but remained higher for CB NK cells activated with IL-15+IL-18 compared to the other cytokines tested (Fig 4.8 B).

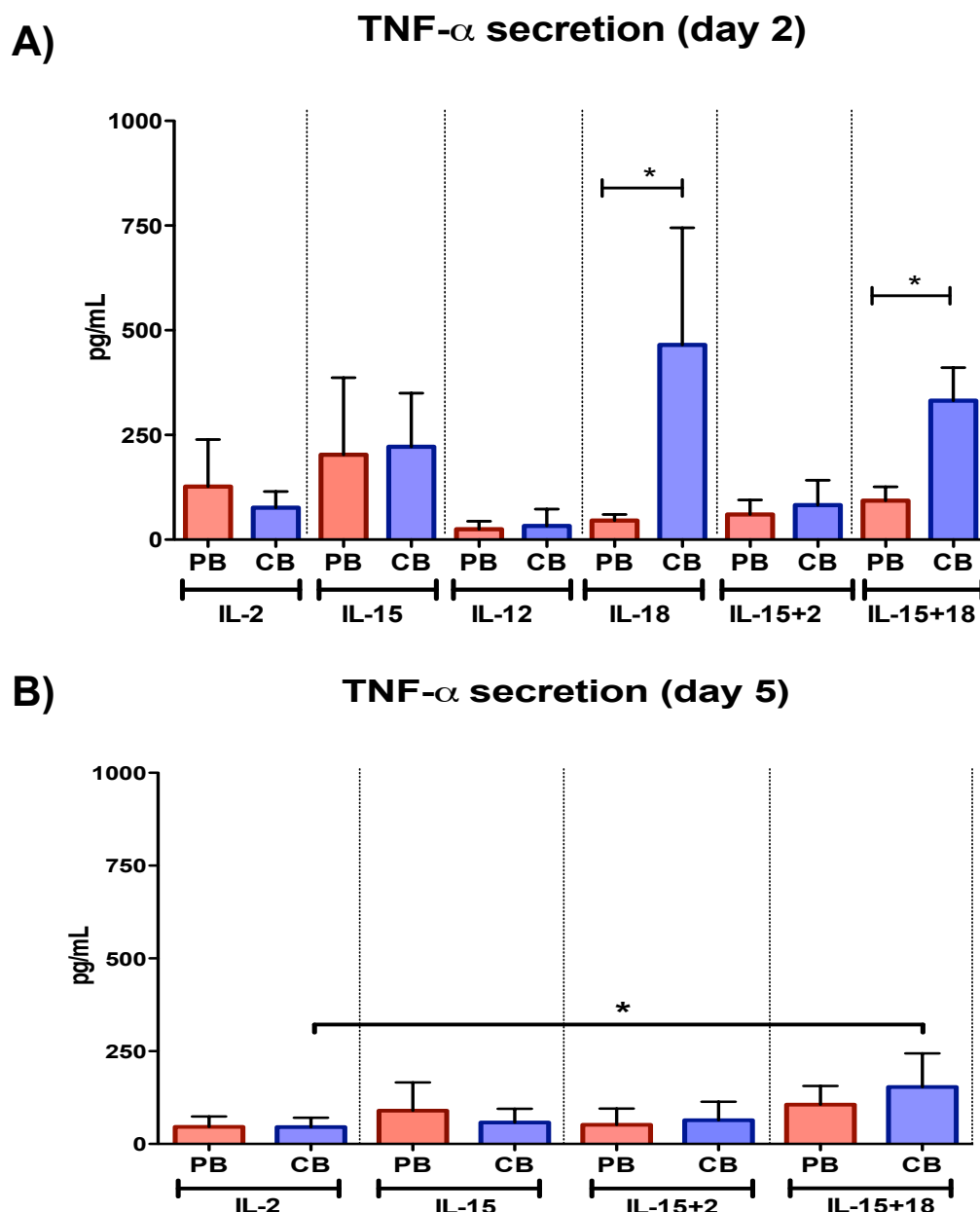


Figure 4.8 TNF- α secretion by cytokine-activated NK cells. Secretion of TNF- α by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. $p < 0.05$ (*). PB N=5, CB N=6.

4.2.3.3 CB NK cells secreted higher levels of TNF- β than PB NK cells at day 5 after stimulation with cytokines

Low secretion of TNF- β was observed for cytokine-activated NK cells at day 2 post-stimulation (Fig 4.9 A). However, it was noticed that the secretion of TNF- β by CB NK cells was increased as compared to PB NK cells 5 days after stimulation (Fig 4.9 B). Significant TNF- β secretion was observed for CB NK cells activated with IL-15 (117.5

± 25.65 pg/mL), IL-15+IL-2 (142.9 ± 43.59 pg/mL), and IL-15+IL-18 (686.5 ± 247.0 pg/mL, $p = 0.0195$, 0.0320 , and 0.0256 respectively).

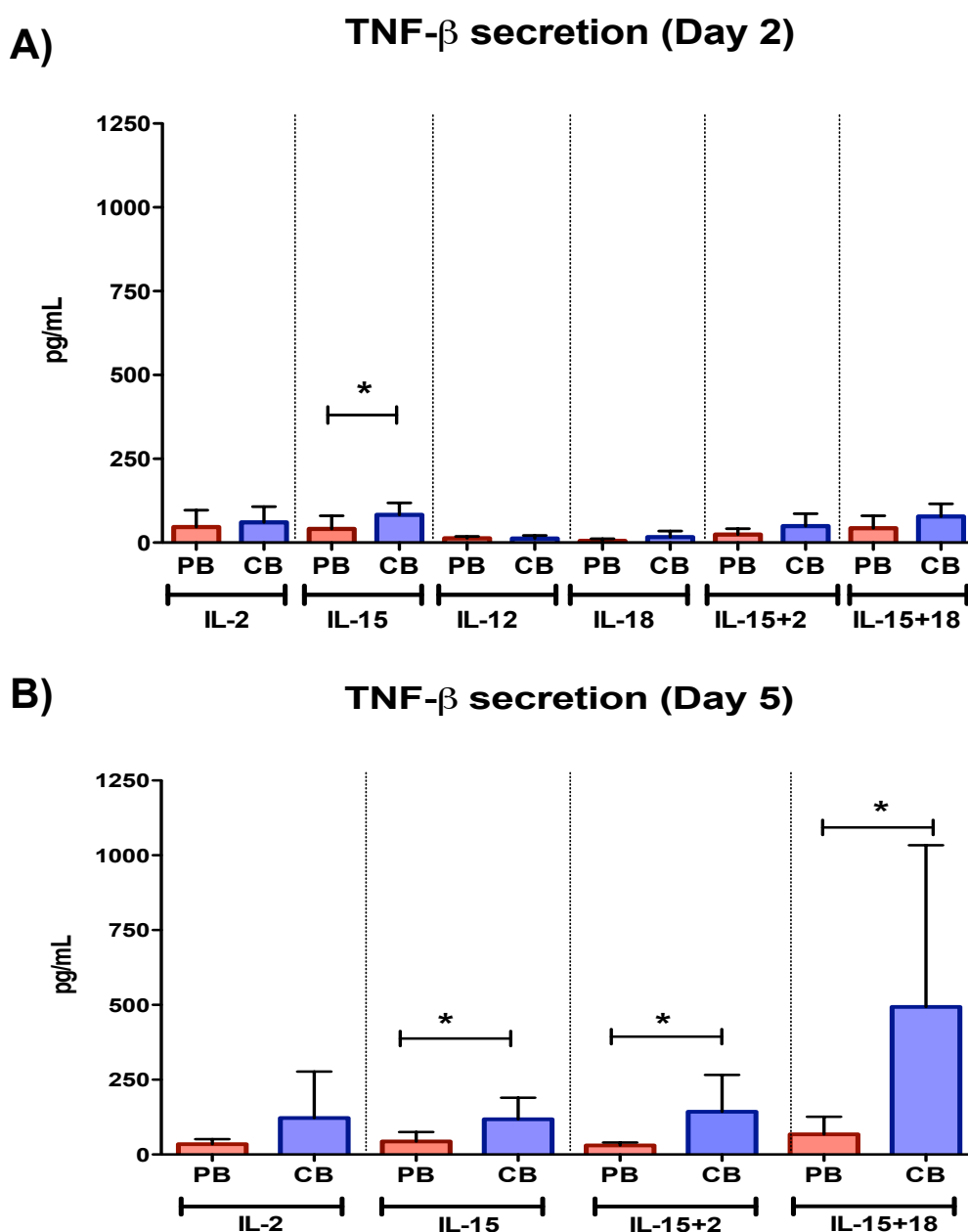


Figure 4.9 TNF- β secretion by cytokine-activated NK cells. Secretion of TNF- β by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. $p < 0.05$ (*). PB N=5, CB N=6.

4.2.3.4 Cytokine-activated NK cells secreted considerable amounts of IL-1 β

IL-1 β is a pro-inflammatory cytokine produced by activated macrophages that acts as a co-stimulator for NK cell proliferation, cytotoxicity and cytokine production (Cooper et

al., 2001b). However, NK cells and B cells also produce IL-1 β (Dinarello, 2009). Variable levels of IL-1 β secretion by both PB NK cells and CB NK cells were detected 2 days after stimulation (Fig 4.10 A). Interestingly, whilst the secretion of IL-1 β by PB NK cells decreased 5 days after cytokine activation, CB NK cells still secreted higher levels of IL-1 β at this time point (Fig 4.10 B).

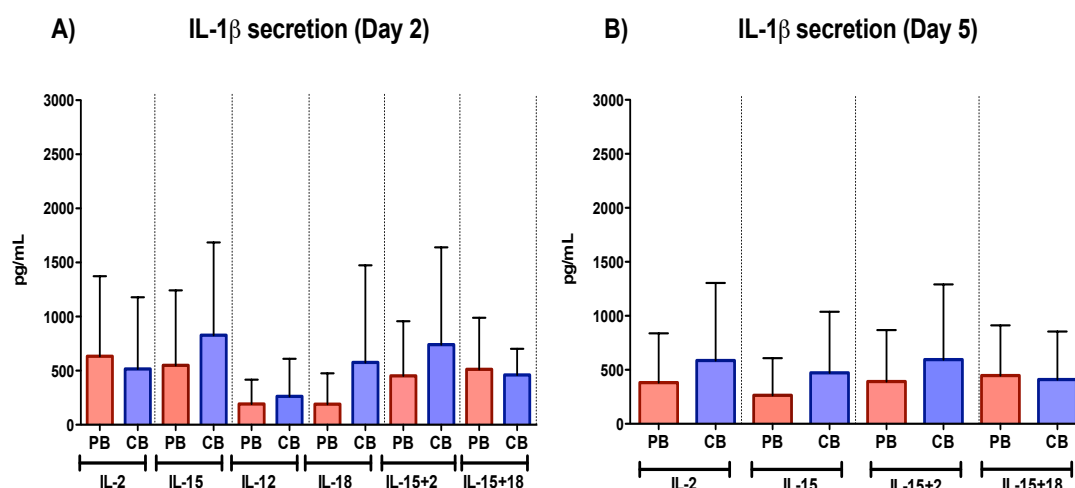


Figure 4.10 IL-1 β secretion by cytokine-activated NK cells. Secretion of IL-1 β by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. PB N=5, CB N=6.

4.2.3.5 CB NK cells secrete higher levels of IL-6 than PB NK cells upon cytokine activation

Different levels of IL-6 secretion by cytokine-activated PB and CB NK cells were detected (Fig 4.11). Both CB and PB NK cells secreted considerable amounts of IL-6 2 days after stimulation with all cytokines tested, yet a trend towards higher secreted IL-6 by CB NK cells was observed, especially following stimulation with IL-2, IL-15, IL-18, IL-15+IL-2 and IL-15+IL-18 (Fig 4.11 A). Interestingly, while IL-6 secretion by cytokine-activated PB NK cells declined at day 5 after activation, CB NK cells persistently produced IL-6 at this time point (Fig 4.11 B).

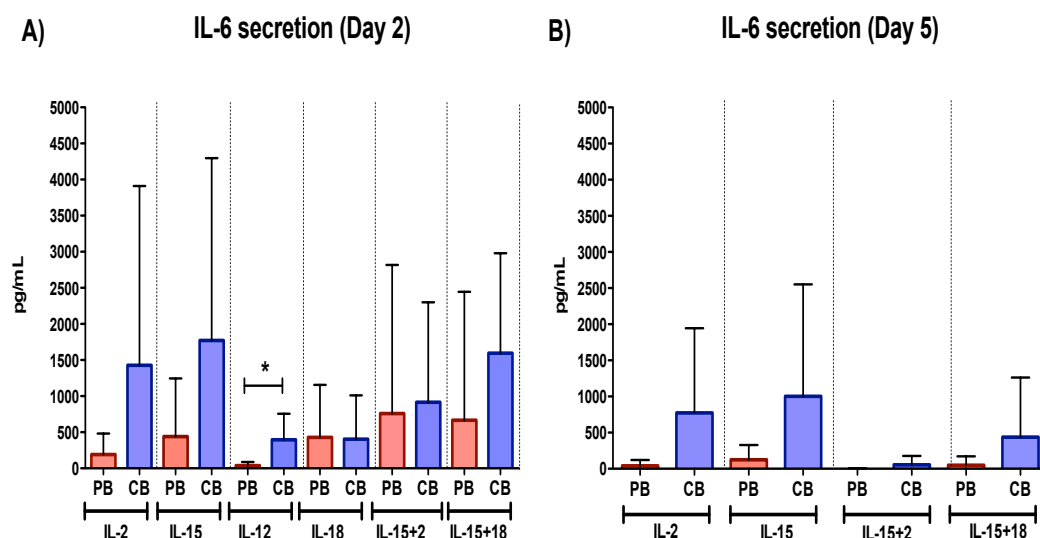


Figure 4.11 IL-6 secretion by cytokine-activated NK cells. Secretion of IL-6 by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. $p < 0.05$ (*). PB N=5, CB N=6.

4.2.3.6 CB and PB NK cells secrete high amounts of IL-8 upon stimulation with cytokines

IL-8 is a T cell recruiting chemokine produced mainly by macrophages, however it has also been shown that NK cells produce IL-8 under certain stimulation conditions (Roda et al., 2006). Both cytokine-activated PB and CB NK cells secreted IL-8 at day 2 after stimulation in all tested conditions (Fig 4.12 A), however IL-8 secretion by PB NK cells was reduced by day 5 (Fig 4.12 B). Upon IL-2 stimulation, CB NK cells secreted significantly higher amounts of IL-8 (3983 ± 1015 pg/mL) than PB NK cells (301.6 ± 130.4 pg/mL, $p = 0.0098$). Similarly, CB NK cells secreted higher amounts of IL-8 upon stimulation with IL-15+IL-2 and IL-15 than PB NK cells activated with the same cytokine.

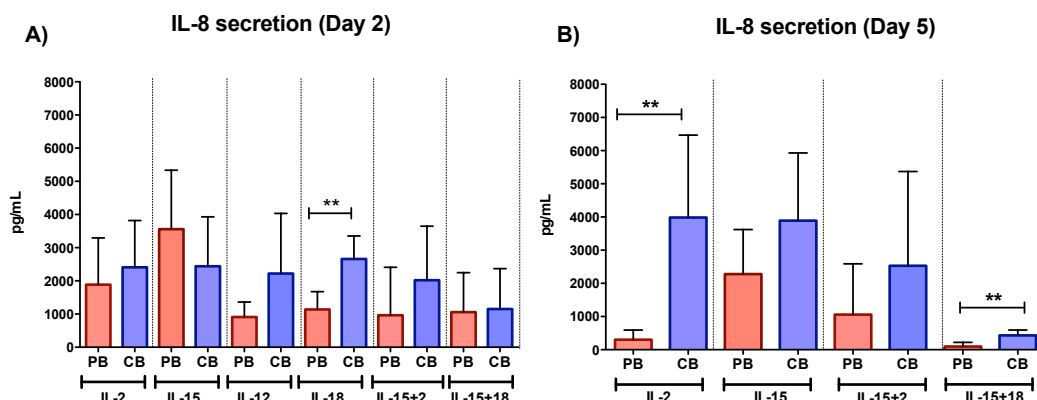


Figure 4.12 IL-8 secretion by cytokine-activated NK cells. Secretion of IL-8 by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. $p < 0.01$ (**). PB N=5, CB N=6.

4.2.3.7 Cytokine-activated NK cells secreted negligible amounts of IL-10 and IL-5, whereas IL-4 secretion was completely undetectable

Although NK cells mainly produce type I cytokines, studies have shown that NK cells can also produce type II cytokines such as IL-5 and IL-10, which act as immunoregulatory cytokines under certain conditions (Cooper et al., 2001c, Warren et al., 1995). Cytokine-activated NK cells secreted very low levels of IL-5 (Fig 4.13). In contrast, IL-4 secretion by PB and CB NK cells was completely undetectable at both time points after stimulation with cytokines. In addition, it was also found that cytokine-activated NK cells produce very low amounts of IL-10 (Fig 4.14).

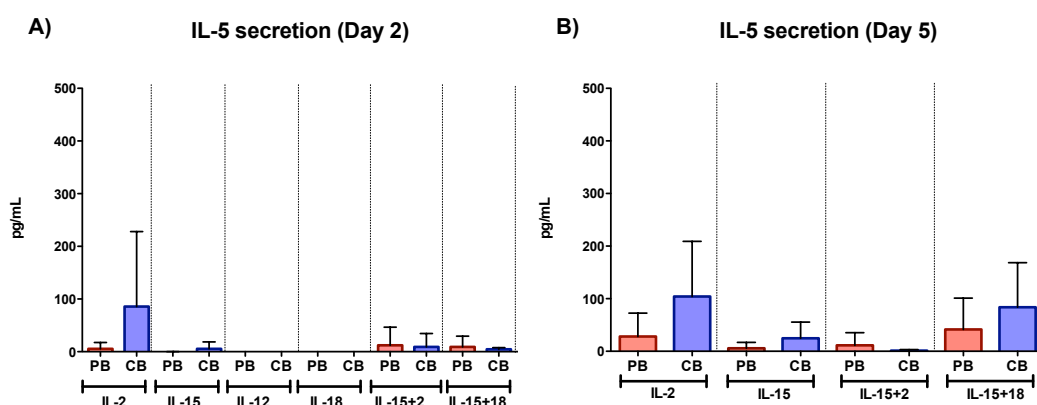


Figure 4.13 IL-5 secretion by cytokine-activated NK cells. Secretion of IL-5 by cytokine-activated PB (red) and CB (blue) NK cells assessed using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. PB N=5, CB N=6.

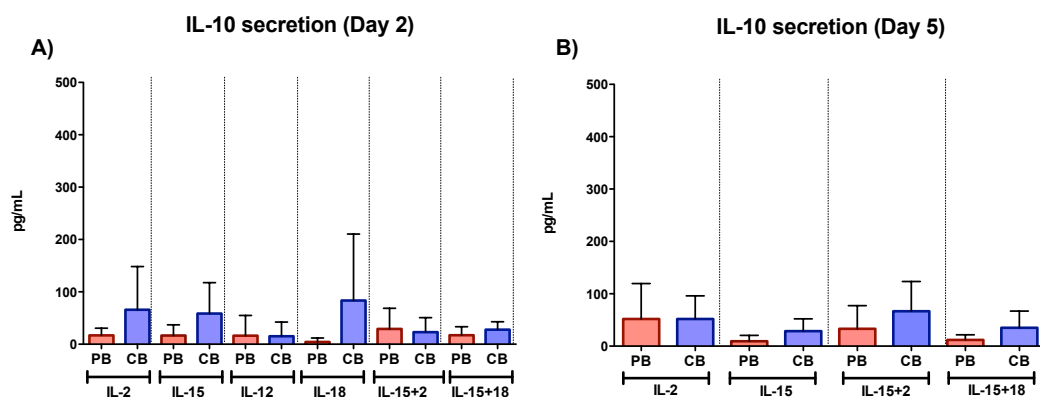


Figure 4.14 IL-10 secretion by cytokine-activated NK cells. Secreted IL-10 by cytokine-activated PB (red) and CB (blue) NK cells tested using multiplex bead-based immunoassay 2 (A) and 5 (B) days after stimulation with cytokines. Results are presented as Mean \pm SD. PB N=5, CB N=6.

4.3 Discussion

CB NK cells have been reported to exhibit low cytotoxicity and reduced IFN- γ production as compared to PB NK cells (Dalle et al., 2005, Gaddy et al., 1995, Luevano et al., 2012a, Xing et al., 2010). Herein, my results show that upon stimulation with cytokines, CB NK cells are responsive to cytokine activation and exhibit proliferative capacity, cytotoxicity and cytokine secretion. However, differential findings were observed based on the type of cytokine used.

I showed that cell proliferation was consistent at day 2 post-stimulation for all cytokines tested for both PB and CB NK cells. In line with published models of proliferation, there is a time delay observed before the first cellular division, regardless of the dose of IL-2 or IL-15 used (Deenick et al., 2003, Zhao and French, 2012). However, higher recruitment of non-dividing cells into replicating cycles was observed by increasing the concentration of cytokines. By day 5, NK cells showed distinct responses towards different cytokines. CB NK cells proliferated more significantly in response to IL-15+IL-18 or IL-15+IL-2, whereas stimulation with IL-2 alone was not sufficient to induce further proliferation at this time point. However, it is noteworthy that CB NK cells could be activated with higher doses of IL-2 (1000 IU/mL), whereas PB NK cells were activated with lower doses of IL-2 (200 IU/mL) and expanded better in response to IL-2 stimulation than CB NK cells. Experiments that aimed to determine the optimal IL-2 concentration required to expand CB NK cells have previously shown that higher doses of IL-2 (500 or 1000 IU/mL) caused significant expansion of NK cells at day 18 (Condiotti et al., 2001). Hence, this finding suggests that CB NK cells need additional time to proliferate in response to IL-2, possibly due to their naivety. PB and CB NK cells favourably proliferated in response to IL-15+IL-18 stimulation. These results are in line with the study of Nomura *et al.* who showed that CB NK cells respond better to IL-18 stimulation than PB NK cells (Nomura et al., 2001). Although it is known that IL-18 alone induces apoptosis of NK cells, this effect was shown to be inhibited by adding high doses of IL-2 resulting in NK cell expansion (Huang et al., 2010). Hence, it could be that the addition of IL-15, which shares many biological similarities with IL-2, reduces the apoptotic effect of IL-18 and promotes subsequent expansion.

I showed that the use of different cytokine conditioning regimes to stimulate NK cells induced different cytotoxic effect against K562 cells by PB and CB NK cells. The cytotoxicity of IL-2 activated CB NK cells was low at day 2, but was enhanced at day 5 after IL-2 stimulation. In contrast, the cytotoxicity of CB NK cells was enhanced at day 2 when stimulated with IL-15 or IL-15+IL-2. IL-15 induces the potent cytotoxic effect of NK cells by upregulating NKG2D, FasL, perforin and TRAIL (Özdemir et al., 2005, Zhang et al., 2008). One study carried out by Satwani and colleagues revealed that the activation of CB NK cells with fms-like tyrosine kinase-3 (FLT-3) + anti-CD3 monoclonal antibodies + IL-2 + IL-15 significantly promoted enhanced killing of Daudi and K562 cell lines, as compared to those activated with similar conditions, but without the addition of IL-15 (Satwani et al., 2011). Furthermore, Zhang *et al.* reported higher cytotoxicity of CB NK cells against K562 and Jurkat cell lines after stimulation with the combination of IL-15+IL-2 exceeding those stimulated with IL-2 alone (Zhang et al., 2011). However, mechanisms underlying the maximal cytotoxicity of IL-15+IL-2 activated CB NK cells remained to be clarified.

It was notable that at day 2, CB NK cells activated with IL-12 or IL-15+IL-18 had lower cytotoxicity than PB NK cells activated with similar conditions. Interestingly, this was associated with substantial production of IFN- γ . It was shown here that the killing capacity of IL-15+IL-18 activated CB NK cells improved as the level of IFN- γ production began to decrease at day 5.

Resting and cytokine-activated CB and PB NK cells expressed granzyme B to different extents. Interestingly, resting CB NK cells demonstrated lower expression of granzyme B than PB NK cells. However, CB NK cells had slightly increased granzyme B expression following activation with all cytokines except IL-18. This was associated with poor killing capacity for CB NK cells activated with IL-18. As NK cell cytotoxicity was measured here using a 4 hour 51 Chromium-release assay, CB NK cells were not able to utilise a FasL-dependent killing pathway which is known to require 12 hours to take effect (Arase et al., 1995). Furthermore, it has been previously shown that NK cells activated with IL-18 do not increase their expression of perforin or granzyme B mRNA (Hyodo et al., 1999). Similarly, I showed here that IL-18 activated CB NK cells have similar levels of perforin to those activated with other cytokines, but very low levels of granzyme B as compared to other cytokine-activated NK cells.

PB NK cells produce substantial amounts of IFN- γ upon stimulation with IL-2 that was associated with elevated levels of IFN- γ mRNA. In contrast to stimulation with IL-2, CB NK cells stimulated with IL-12, IL-15 or IL-15+IL-18 selectively secreted high quantities of IFN- γ . It is likely that abundant secretion of IFN- γ by CB NK cells in response to IL-15, IL-12 or IL-15+IL-18 is due to the fact that these cytokines are ubiquitous in early immune inflammatory responses. The lower responsiveness of CB NK cells to IL-2 stimulation as compared to other cytokines could be due to their naivety. Indeed, IL-2 is a product of activated T cells and since the majority of CB T cells are characterised by a CD45RA⁺ phenotype, it is less likely that CB NK cells have been exposed to IL-2. Instead, CB NK cells theoretically should be readily responsive to innate inflammatory cytokine such as IL-18, as Nomura and colleague have suggested (Nomura et al., 2001).

In addition to IFN- γ , NK cells produce another potent cytokine, TNF- α . Quiescent NK cells constitutively express trans-membrane TNF- α . Upon stimulation, trans-membrane TNF- α is converted into soluble TNF- α that plays a major role in antiviral and antitumor immunity (Caron et al., 1999). Blocking of TNF- α expressed by CB NK cells using monoclonal antibodies affected their survival, proliferation and the development of a mature phenotype following cytokine stimulation (Gaddy and Broxmeyer, 1997). Resting CB NK cells express significantly less intracellular TNF- α than PB NK cells (Krampera et al., 2000). However, it was shown here that CB NK cells secreted greater amounts of TNF- α , particularly upon stimulation with IL-18 or IL-15+IL-18, exceeding the amount produced by PB NK cells under similar conditions. IL-18 stimulation mediates the production of TNF- α , but inversely affected NK cell viability. Blocking experiments showed that IL-18-mediated NK cell apoptosis is partially prevented by the addition of anti-TNF- α (Shibatomi et al., 2001). However, TNF- α production here appeared to be potentiated by the addition of IL-15, which was also associated with a high proliferative capacity of CB NK cells activated with IL-15+IL-18. Correspondingly, it was shown previously that endogenous TNF- α is essential for NK cell proliferation (Naume et al., 1991). Hence, the substantial proliferation of IL-15+IL-18 activated CB NK cells observed here is likely to be influenced by elevated production of TNF- α .

Another member of the TNF family is TNF- β , which is a highly inducible protein also known as lymphotoxin- α (LT α). TNF- β is secreted by activated macrophages and lymphocytes, and promotes the phagocytosis of infected cells, as well as T helper cell differentiation (Falvo et al., 2013). NK cells, typically CD56^{bright} NK cells, secrete large amounts of TNF- β after stimulation with IL-15 and IL-18 (Cooper et al., 2001c). Interestingly, TNF- β secreted by NK cells results in inhibition of viral replication in a non-cytolytic dependent manner (Iversen et al., 2005). In addition, TNF- β play a key role during NK cell differentiation and maturation as *Lta*^{-/-} (TNF- β -deficient) knockout mice have shown reduced NK cell number and defective cytotoxicity (Iizuka et al., 1999). Herein, it was shown that TNF- β secretion by PB and CB NK cells was low at day 2 after stimulation with cytokines. However, by day 5, CB NK cells demonstrated higher secretion of TNF- β than PB NK cells, especially when stimulated with the combination of IL-15+IL-18. Together, these data support the hypothesis that the combination of IL-15 with IL-18 potently promotes cytokine secretion by CB NK cells. Yet, those cells still have the capacity to kill their target by exocytosis as shown using ⁵¹Cr-release assays.

IL-1 β is a pro-inflammatory cytokine mainly produced by activated monocytes which provides a co-stimulatory signal for NK cells to produce IFN- γ (Cooper et al., 2001b). It has been reported that NK cells secrete a modest amount of IL-1 β following priming with IL-2 (De Sanctis et al., 1997). Here I showed that all cytokine conditions tested resulted in the production of IL-1 β by NK cells. However, these results should be confirmed by intracellular staining, as IL-1 β secretion was variable from sample to sample. Similarly, many immune cells including neutrophils and APC produce IL-6 during early inflammatory responses and lymphocytes produce IL-6 during late immune responses (Hirano, 1992). NK cells were shown to promote the production of IL-6 by DCs and macrophages. However, few reports showed that NK cells directly secrete IL-6 (Horikawa et al., 2005, Schmidt et al., 2004). It has also been shown that the production of IL-6 by NK cells is crucial to shape T helper immune response after vaccination (Hall et al., 2010). I did observe IL-6 production by NK cells at day 2 post-stimulation with cytokines. However, CB NK cells still produce IL-6 at day 5 post-stimulation, whereas PB NK cells did not produce any IL-6 beyond day 2. Condiotti and colleagues have shown that expanded CB NK cells produced significantly higher levels of IL-6 than PB NK cells after 10 days of stimulation with IL-2. These cells were

expanded using an autologous lymphocyte feeder layer. Thus, it is unknown whether the IL-6 production in the Condiotti study is a result of NK cell interaction with the feeder layer or cytokine stimulation (Condiotti et al., 2001). Despite this, herein, it is clearly demonstrated that IL-6 secretion by NK cells could be induced by cytokine activation alone.

IL-8 is a neutrophil chemotactic factor produced by macrophages and epithelial cells which enhances the trafficking of leucocytes, including CD56⁺ NK cells, to inflammatory sites. Herein, cytokine-activated NK cells secreted high levels of IL-8 2 days after stimulation with cytokines. At day 5 of cytokine activation, it was noticeable that IL-8 secretion by PB NK cells decreased, however, CB NK cells secreted higher quantities of IL-8 at this time point. The secretion of IL-8 by NK cells has been observed under certain conditions including stimulation with IL-15 (El-Shazly and Lefebvre, 2011), IL-2 or IL-12 in the presence of breast cancer tumour cells (Roda et al., 2006), NK cell following purification in the presence of FCS and after stimulation with IL-2 plus anti-CD16 (Somersalo et al., 1994). Moreover, IL-8 is abundantly produced by dNK cells and is associated with poor lytic function of these cells (Saito et al., 1994, Vacca et al., 2008). Herein, my results demonstrate that both PB and CB NK cells produced IL-8 abundantly with a trend toward higher secretion by CB NK cells. The observation of a high production of IL-8 could be due to culture conditions, as it was shown that the addition of FCS to purified NK cells induces IL-8-mediated chemotaxis in contrast to those supplemented with human serum albumin (Somersalo et al., 1994). Yet, CB NK cells persistently produce IL-8 that could be correlated with their immaturity.

It is believed that under certain conditions NK cells mediate immunoregulatory responses regulated by cytokine secretion (Cooper et al., 2001c). IL-10 is an immunoregulatory cytokine secreted by T cells, B cells and monocytes upon stimulation with GM-CSF, IFN- γ , IL-1, IL-12 or TNF- α . The secretion of IL-10 inhibits antigen-specific proliferation and downregulates the synthesis of inflammatory cytokines (Del Prete et al., 1993, Howard and O'Garra, 1992). Although earlier studies could not detect IL-10 production by NK cells (Carson et al., 1995, Spagnoli et al., 1993), subsequent findings have shown that NK cells secrete low amounts of IL-10 upon stimulation with IL-2, but not following stimulation with IL-12 (Mehrotra et al.,

1998). Furthermore, NK cells express both IL-10 mRNA and protein intracellularly (Mehrotra et al., 1998). Cooper *et al.* showed that CD56^{bright} NK cells produce substantial amounts of IL-10 upon stimulation with IL-15 and IL-12 (Cooper et al., 2001c). Herein, I showed that cytokine-activated PB and CB NK cells secreted low levels of IL-10. It is worth noting that these analyses were carried out by multiplex bead array. Reports aiming to evaluate multiplex performance in comparison to traditional ELISA showed greater sensitivity of multiplex for detection of small amounts of secreted cytokines (duPont et al., 2005, Elshal and McCoy, 2006).

NK cells accumulate at inflammatory sites and initiate interactions with other innate immune cells by releasing cytokines. IL-5, produced by NK cells, mediates T_H2 responses through facilitation of mobility and differentiation of eosinophils. In these studies, production of IL-5 by NK cells appeared to be regulated by cytokine stimulation (Warren et al., 1995). IL-2 was shown to promote IL-5 secretion, whereas its secretion was inhibited by IL-12. Herein, I showed that PB NK cells produced negligible amounts of IL-5, however, a trend towards higher IL-5 production was detected for CB NK cells. It was shown that NK cells at intermediate stages of differentiation produce type-II cytokines, including IL-5 and IL-13. Nonetheless, progression towards final stages of NK cell maturation is associated with loss of their ability to produce these cytokines and subsequent acquisition of an IFN- γ ⁺ phenotype (Loza and Perussia, 2001, Loza et al., 2002). Hence, secretion of IL-5 by CB NK cells is suggestive of a unique cytokine production profile that is possibly correlated with their maturation stage.

To summarise, cytokine activation induces fully functional CB NK cells with differential findings based on the cytokine conditioning used. While IL-2 seems to be an ideal cytokine to stimulate PB NK cells, other cytokines promote unprecedented functional capacities of CB NK cells. IL-15 or the combination of IL-15+IL-2 promote enhanced killing of K562 cells by CB NK cells whereas the combination of IL-15+IL-18 selectively promotes proliferation and inflammatory cytokine secretion of CB NK cells. Collectively, IL-15 appears to be crucial to promote the functionality of CB NK cells. In addition, the cytokine secretion repertoire of CB NK cells is shown to be distinct from PB NK cells and could be associated with their degree of maturity. Herein, CB NK cells showed functional competency post-cytokine activation.

Chapter 5: Analysis of the NK cell homing receptor repertoire

5.1 Introduction

NK cell migration and trafficking is regulated by chemokines (Inngjerdnngen et al., 2001, Kim et al., 1999, Robertson et al., 2000, Taub et al., 1995). At steady state, the majority of NK cells circulate in the periphery as part of their role in immune surveillance. However, a low percentage of NK cells, the CD56^{bright} subset, express chemokine receptors which allow them to traffic to inflamed lymph nodes (LN) (Robertson, 2002). CD56^{bright} NK cells predominantly express a high density of the adhesion molecule L-selectin, and this facilitates their rolling on the high endothelial venules that are present in LN (Frey et al., 1998). Moreover, CD56^{bright} NK cells express CCR7 which binds to CCL19 and CCL21 to promote lymphocyte recruitment to the T cell zone of LN (Kim et al., 1999). NK cell recruitment to draining LN results in significant secretion of IFN- γ and subsequent polarisation of a T helper immune response.

At steady state, resting CD56^{dim} NK cells express CXCR1, CX3CR1 and the Chemerin Receptor 23 (ChemR23), all of which mediate their homing to inflamed non-lymphoid sites in which NK cell effector function is essential (Campbell et al., 2001, Parolini et al., 2007). Accumulation of NK cells at inflammation sites facilitates interaction with APCs such as macrophages and DCs. Subsequently, CD56^{dim} NK cells can acquire LN homing receptors upon activation via inflammatory cytokines such as IL-18 which is produced by APCs (Mailliard et al., 2005), or by receptor uptake via interactions with mature DCs (Marcenaro et al., 2009). Furthermore, activated NK cells upregulate CXCR3 which mediates the potent chemotaxis of NK cells towards its ligands; MIG (CXCL9), interferon- γ -inducible protein 10 (CXCL10) and interferon-inducible-T-cell- α (CXCL11) (Inngjerdnngen et al., 2001, Campbell et al., 2001).

It has previously been shown by our group and others that CB NK cells do not express L-selectin, thereby suggesting that CB NK cells are unable to migrate to draining LN by this mechanism (Dalle et al., 2005, Luevano et al., 2012a). Moreover, resting CB NK

cells were found to have significantly higher expression of CXCR4 and lower expression of CXCR1 than PB NK cells, implying an enhanced capacity of CB NK cells to traffic to the BM, but a lower capacity to be recruited to inflamed sites (Luevano et al., 2012a). Hence, the aim of this study was to assess whether cytokine activation resulted in upregulation of chemokine receptors that mediate NK cell migration to LN, and if so, whether this correlated with increased NK cell chemotaxis *in vitro*.

5.2 Results

5.2.1 Expression of chemokine receptors on resting and cytokine-activated NK cells

Upregulation of CCR7, CXCR3 and L-selectin by NK cells is essential for NK cell trafficking to LN and inflamed sites. Hence, the following analysis investigates upregulation of those chemokine receptors by PB and CB NK cells upon cytokine activation.

5.2.1.1 CB NK cells upregulate L-selectin upon activation with IL-12 or IL-18, whereas resting and cytokine-activated PB NK cells constitutively express L-selectin

It was previously found that resting CB NK cells do not express L-selectin (CD62L) (Dalle et al., 2005, Luevano et al., 2012a). Therefore, the ability to induce L-selectin expression by PB and CB NK cells by cytokine activation was tested. IL-12 induced the highest levels of L-selectin expression by CB NK cells (56.83 ± 3.45 %) and PB NK cells (56.28 ± 3.85 %) (Fig 5.1 A). IL-18 also significantly induced L-selectin expression by both PB NK cells (37.20 ± 1.439 %) and CB NK cells (24.63 ± 2.30 %) (Fig 5.1 A). PB NK cells responded better to IL-2 activation showing significant upregulation of L-selectin (35.78 ± 10.30 %), as compared to CB NK cells (3.44 ± 0.94 %) ($p = 0.0205$). As compared to other cytokines, IL-15 activation induced low levels of L-selectin expression by CB NK cells (Fig 5.1 B). Increasing doses of IL-12 or IL-15 did not further upregulate L-selectin expression by NK cells (Fig 5.2).

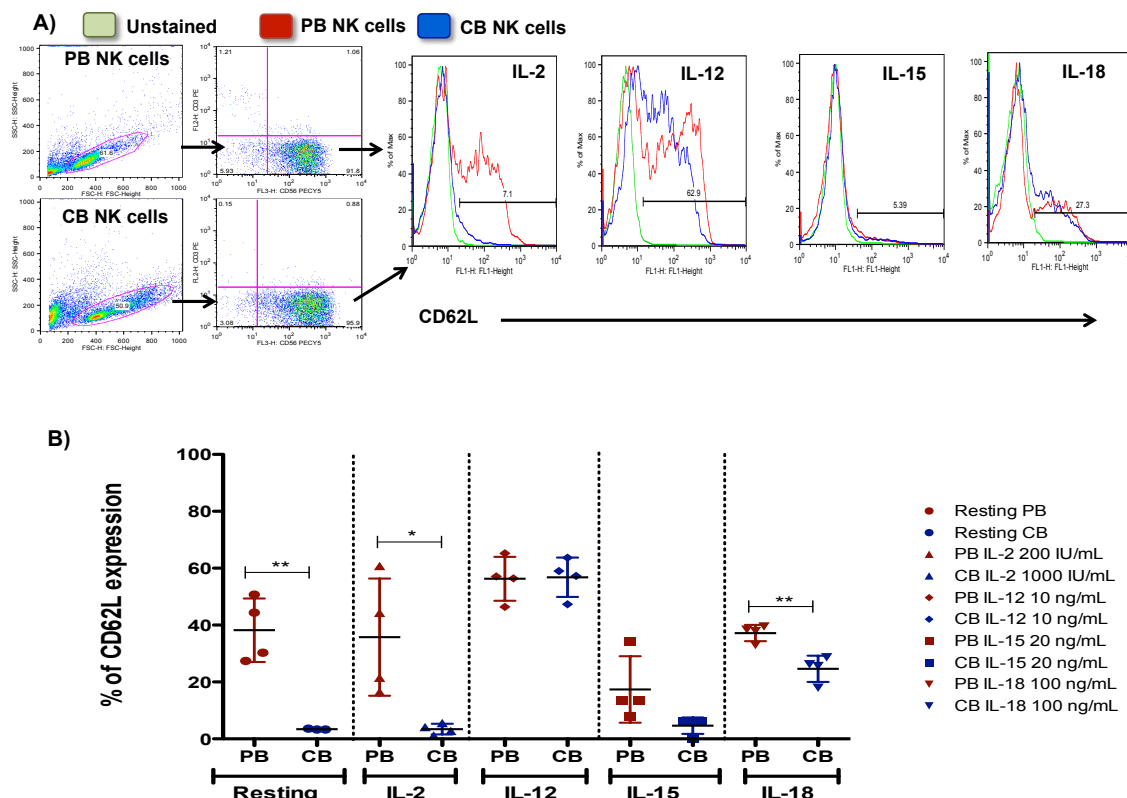


Figure 5.1 Expression of L-selectin (CD62L) by PB and CB NK cells after cytokine stimulation. A) Histograms showing CD62L upregulation in response to cytokine activation. B) Comparison of CD62L expression by resting and cytokine-activated PB and CB NK cells. Analysis was performed on purified NK cells and the expression of CD62L was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*) and $p < 0.01$ (**).

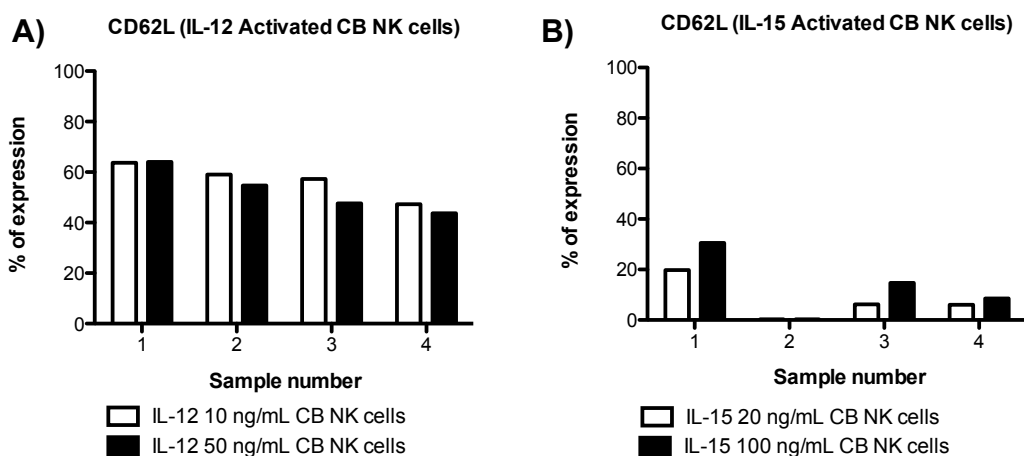


Figure 5.2 Upregulation of L-selectin (CD62L) by CB NK cells after activation with IL-12 and IL-15. L-selectin expression by CB NK cells activated with IL-12 (10 ng/mL or 50 ng/mL) (A) and IL-15 (20 ng/mL or 100 ng/mL) (B). N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

5.2.1.2 CB NK cells upregulate chemokine receptors involved in lymph node homing upon cytokine activation

Here, expression of the LN homing receptors CXCR3 and CCR7 was assessed on CB and PB NK cells following cytokine activation. Upregulation of these two chemokine receptors leads to NK cell recruitment into draining LN, which is critical for shaping the immune response. Herein, it was observed that resting PB and CB NK cells expressed very low levels of CXCR3, however resting PB NK cells exhibited slightly higher CXCR3 expression (1.887 ± 0.28 %) as compared to resting CB NK cells (0.4333 ± 0.05 %, $p = 0.0073$) (Fig 5.3 A, B). Activation with all the cytokines tested resulted in significant upregulation of CXCR3 by PB and CB NK cells (Fig 5.3 B). A unique pattern of expression was observed when CB NK cells were activated with IL-2 and IL-12, in that induced a significantly higher level of CXCR3 expression (92.10 ± 2.81 % and 72.50 ± 13.70 % respectively) as compared to that on PB NK cells (67.75 ± 9.01 % and 25.40 ± 5.11 % respectively). The highest levels of CXCR3 expression by PB (97.30 ± 0.93 %) and CB (96.18 ± 1.62 %) NK cells were observed following IL-15 activation. No detectable change in CXCR3 expression was observed when the concentrations of IL-12 or IL-15 were increased (Fig 5.4).

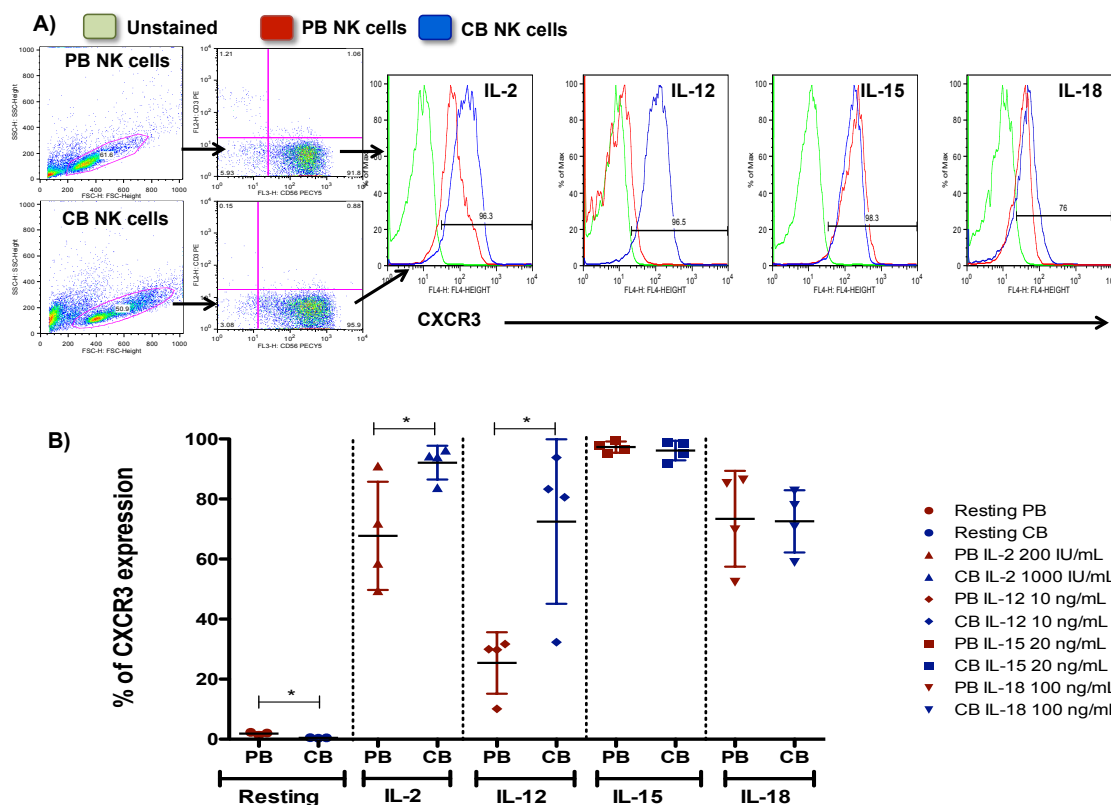


Figure 5.3 Expression of CXCR3 by PB and CB NK cells after cytokine activation. A) CXCR3 upregulation by PB and CB NK cells in response to activation with cytokines. B) Comparison of CXCR3 expression by resting and cytokine-activated NK cells from PB and CB. Analysis was performed on purified NK cells and the expression of CXCR3 was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*).

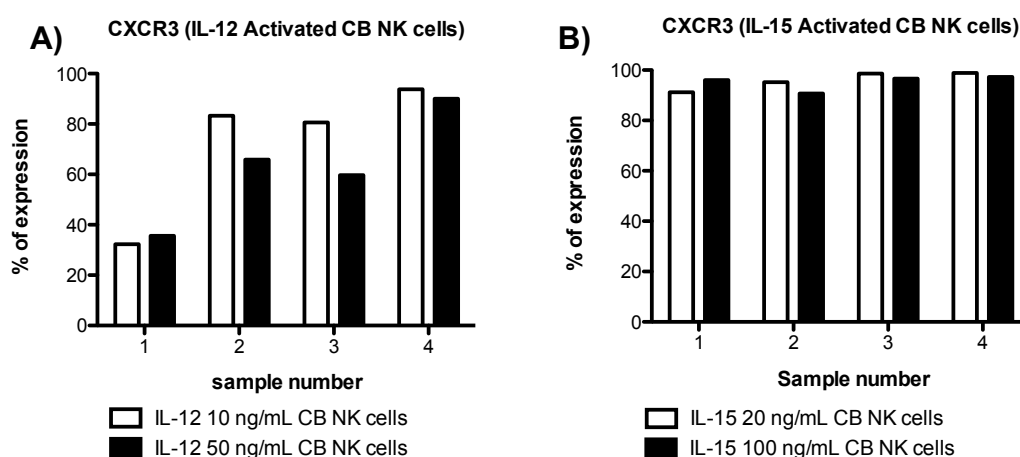


Figure 5.4 Upregulation of CXCR3 after activation with IL-12 and IL-15. CXCR3 expression by CB NK cells activated with IL-12 (10 ng/mL or 50 ng/mL) (A) and IL-15 (20 ng/mL or 100 ng/mL) (B). N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

Expression of CCR7 was difficult to consistently detect, as a consequence of staining for CCR7 was performed using two different monoclonal antibody clones, one from BD Biosciences (clone 3D12) (Fig 5.5 A) and the other one from R&D Systems (clone 358426) (Fig 5.5 B). Variable CCR7 expression by NK cells was observed using both monoclonal antibodies. Notably, the CCR7 clone from R&D Systems detected higher CCR7 expression than the clone from BD Biosciences. Figure 5.6 shows CCR7 expression on CB NK cells with different doses of IL-12 and IL-15.

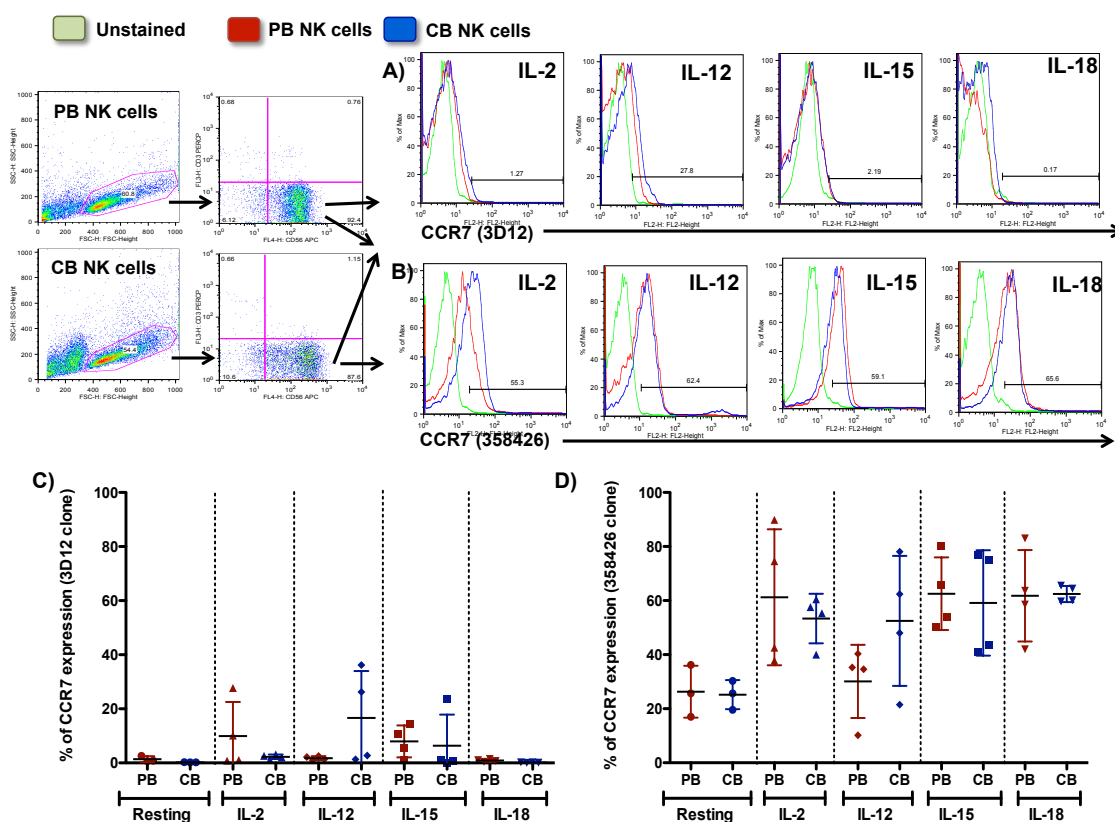


Figure 5.5 Expression of CCR7 by PB and CB NK cells. A) Flow cytometry histograms showing CCR7 expression by cytokine-activated NK cells using the clone 3D12 from BD Biosciences. B) Flow cytometry histograms showing CCR7 expression by cytokine-activated NK cells using the clone 358426 from R&D Systems. C and D) Comparison of CCR7 expression by resting and cytokine-activated NK cells from CB and PB using the 3D12 clone from BD Biosciences and the 358426 clone from R&D Systems respectively. Analysis was performed on purified NK cells and the expression of CCR7 was examined 2 days post IL-12 or IL-18 stimulation, and 5 days post IL-2 or IL-15 stimulation. Results are presented as Mean \pm SD. N=4.

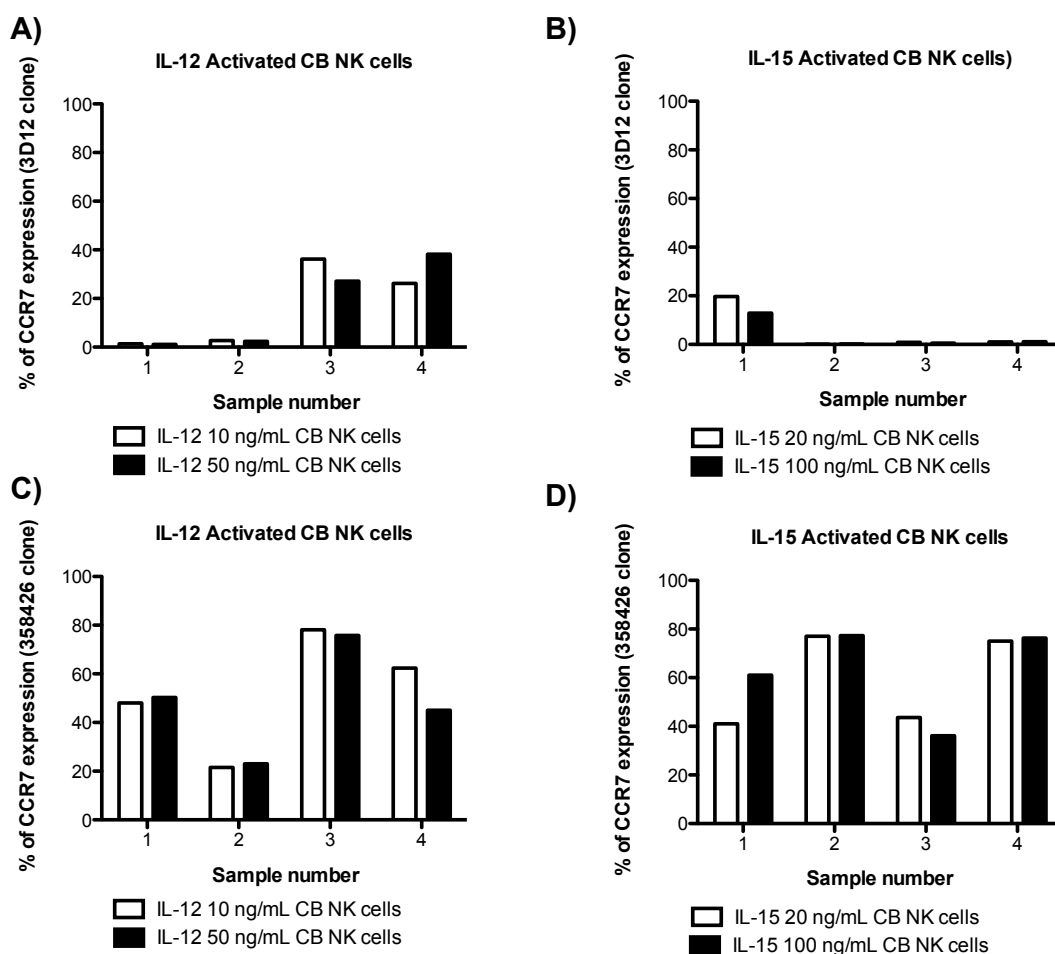


Figure 5.6 Upregulation of CCR7 by CB NK cells after activation with IL-12 and IL-15. CCR7 expression by CB NK cells activated with 10 or 50 ng/mL IL-12 (A) and 20 or 100 ng/mL IL-15 (B) tested using the 3D12 clone from BD Biosciences. (C) CCR7 expression by CB NK cells activated with 10 or 50 ng/mL IL-12 (D) and 20 or 100 ng/mL IL-15 tested using the 358426 clone from R&D Systems. N=4. Analysis was performed on purified NK cells stimulated with IL-12 for 2 days or IL-15 for 5 days.

5.2.2 Analysis of NK cell migratory capacity using trans-well migration assay

The following analysis aimed to assess the migratory capacity of resting or cytokine-activated NK cells towards LN chemoattractants CCL19/21 or CXCL10/12 *in vitro*. Migrated NK cells in response to chemokines were collected from the lower chamber of trans-well plates and the percentage of migration was calculated as (number of migrated cells ÷ positive control)×100. The expression of the chemokine receptors, CCR7 and CXCR3, which are involved in recruitment of NK cells towards these chemoattractants, was also assessed.

5.2.2.1 PB and CB NK cells activated with IL-15+IL-2 exhibit significant migratory capacity in response to CCL19/21 or CXCL10/11

Comparable mean percentage of migration towards CCL19/21 was detected for resting PB NK cells (13.09 ± 1.25 %) and resting CB NK cells (16.15 ± 2.82 %) (Fig 5.7 A). Activation with IL-15+IL-2 induced significant migration of NK cells in response to CCL19/21. The mean percentage of migration by IL-15+IL-2 activated PB NK cells was 57.35 ± 8.52 %, whereas the mean percentage of migration by IL-15+IL-2 activated CB NK cells was 42.66 ± 9.37 % (Fig 5.7 A). Correspondingly, it was found that CCR7 was significantly upregulated on PB and CB NK cells upon stimulation with IL-15+IL-2 (Fig 5.7 B). IL-15+IL-2 activated CB NK cells showed significantly higher mean percentage of CCR7 expression (16.33 ± 2.39 %) than PB NK cells (8.388 ± 0.97 %, $p = 0.0220$).

In addition, IL-15+IL-2 activation promoted considerable CB NK cell migration towards CXCL10/11, as compared to resting NK cells (Fig 5.7 C). While resting PB NK cells showed percentage of migration towards CXCL10/11 of 15.70 ± 2.27 %, IL-15+IL-2 activated PB NK cells exhibited up to 59.88 ± 5.80 % migration ($p = 0.0004$). Similarly, resting CB NK cells showed percentage of migration towards CXCL10/11 of 11.28 ± 3.25 %, whereas IL-15+IL-2 activated CB NK cells showed 47.71 ± 9.35 % migration ($p = 0.0026$). NK cell migration in response to CXCL10/11 was associated with substantial upregulation of CXCR3 by IL-15+IL-2 activated PB and CB NK cells (Fig 5.7 D). Notably, stimulation with IL-15+IL-2 substantially upregulated CXCR3 by PB NK cells (89.05 ± 1.23 %), as well as by CB NK cells (90.25 ± 0.912 %, $p < 0.0001$).

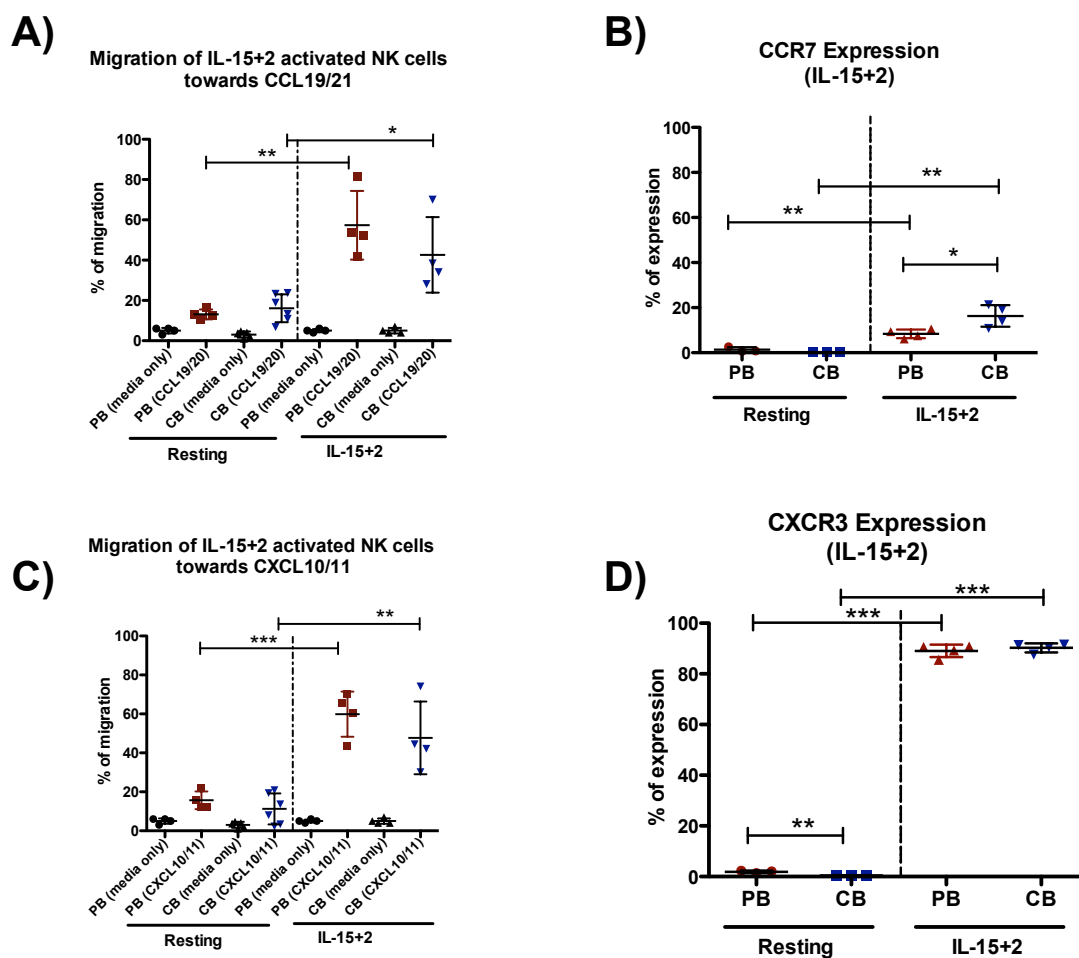


Figure 5.7 Migration of IL-15+IL-2 activated NK cells in response to lymph node chemoattractants. A) Percentage of migration of PB and CB NK cells towards CCL19/CCL21 by both resting and IL-15+IL-2 activated NK cells. B) CCR7 expression by NK cells activated with IL-15+IL-2 as compared to resting NK cells. C) Percentage of migration of PB and CB NK cells towards CXCL10/11 by both resting and IL-15+IL-2 activated NK cells. D) CXCR3 expression by NK cells activated with IL-15+IL-2 as compared to resting NK cells. This analysis was performed on purified NK cells 2 days post IL-15+IL-2 stimulation. Migrated cells were collected from the lower chamber of the trans-well plate and the percentage of migration was calculated as $(\text{no of migrated cells} \div \text{positive control}) \times 100$. Results are presented as Mean \pm SD. $N=4$, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

5.2.2.2 IL-15+IL-12 activation promotes PB NK cell migration, but not CB NK cell migration in response to CCL19/21 or CXCL10/11

The combination of IL-15+IL-12 was included in this study, as it was observed that IL-12 activation induces L-selectin expression by CB NK cells (Fig 5.1). However, trans-well migration assays indicate that whilst activation with IL-15+IL-12 leads to

enhanced PB NK cell migration towards CCL19/21 (34.95 ± 7.24 %), migration of CB NK cells towards CCL19/21 was unchanged (23.50 ± 4.14 %) (Fig 5.8 A). Surprisingly, CCR7 expression on CB NK cells activated with IL-15+IL-12 was significantly upregulated (24.75 ± 3.76 %), as compared to PB NK cells (14.28 ± 1.48 %, $p = 0.041$), which did not translate in an enhanced migratory capacity of CB NK cells (Fig 5.8 B). Similarly, IL-15+IL-12 activation resulted in significantly higher percentage of PB NK cell migration (35.20 ± 3.68 %) in response to CXCL10/11 as compared to CB NK cells (19.90 ± 2.71 %, $p = 0.0155$) (Fig 5.8 C). Correspondingly, this was associated with considerable upregulation of CXCR3 by PB NK cells upon stimulation with IL-15+IL-12 in comparison to CB NK cells (91.43 ± 0.624 % and 73.18 ± 6.027 % respectively, $p = 0.0236$) (Fig 5.8 D).

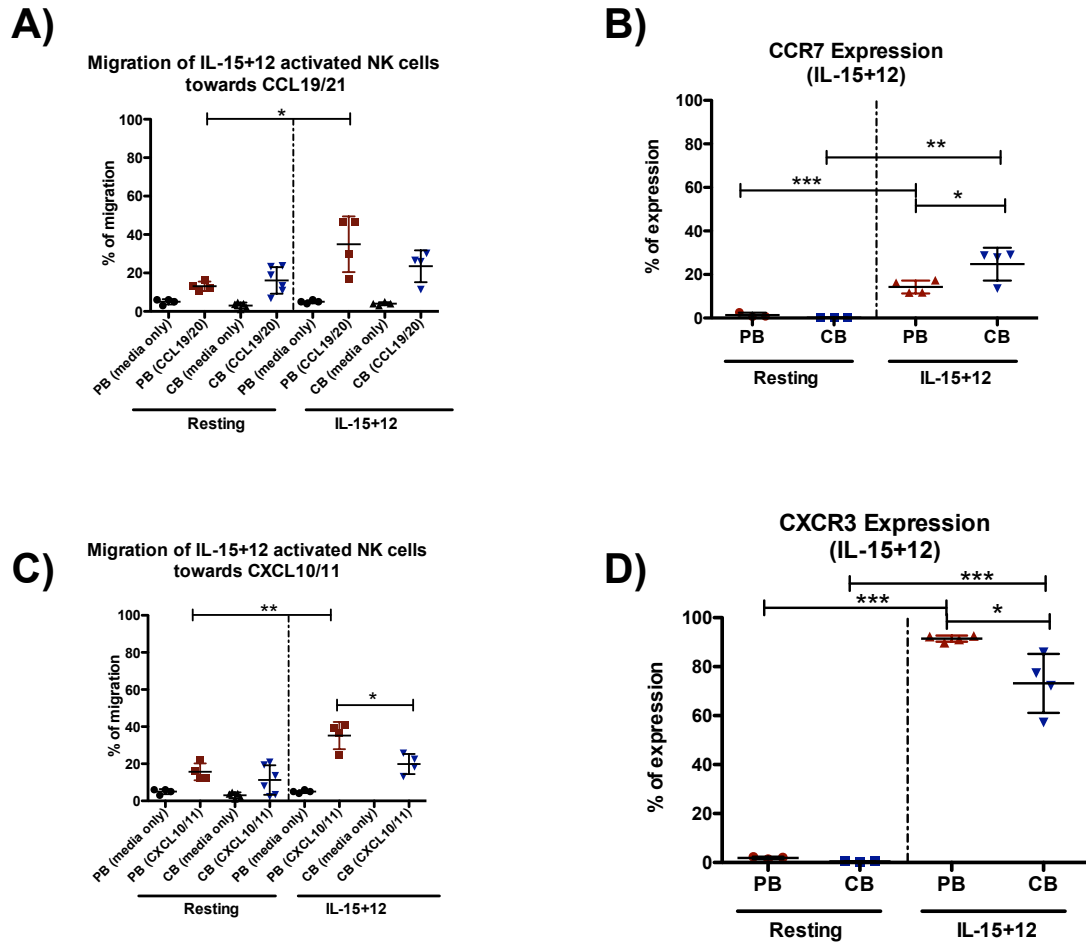


Figure 5.8 Migration of IL-15+IL-12 activated PB and CB NK cells in response to lymph node chemoattractants. A) Percentage of migration of PB and CB NK cells towards CCL19/CCL21 by both resting and IL-15+IL-12 activated NK cells. B) CCR7 expression by NK cells activated with IL-15+IL-12, as compared to resting NK cells. C) Percentage of migration of PB and CB NK cells towards CXCL10/11 by both resting and IL-15+IL-12 activated NK cells. D) CXCR3 expression by NK cells activated with IL-15+IL-12, as compared to resting NK cells. This analysis was performed on purified NK cells 2 days post IL-15+IL-12 stimulation. Migrated cells were collected from the lower chamber of the trans-well plate and the percentage of migration was calculated as (no of migrated cells ÷ positive control)×100. Results are presented as Mean ± SD. N=4, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

5.2.2.3 IL-15+IL-18 activation induces significant migration of PB and CB NK cells in response to CCL19/21 or CXCL10/11

Activation with IL-15+IL-18 promoted significant migration towards CCL19/21 by PB NK cells (38.98 ± 5.393 %) and CB NK cells (52.16 ± 10.15 %), as compared to resting PB and CB NK cells ($p = 0.0034$ and $p = 0.0034$ respectively) (Fig 5.9 A). Upregulation of CCR7 was consistent with the observed migration pattern as both PB

(10.19 ± 1.21 %) and CB (25.35 ± 4.47 %) NK cells significantly expressed CCR7 after stimulation with IL-15+IL-18, as compared to resting PB and CB NK cells ($p = 0.0022$ and $p = 0.0051$ respectively) (Fig 5.9 B).

Similarly, PB and CB NK cells activated with IL-15+IL-18 showed enhanced migration towards CXCL10/11 (Fig 5.9 C). The mean percentage of migrated NK cells in response to CXCL10/11 was 44.36 ± 7.97 % for PB NK cells and 32.32 ± 9.69 % by CB NK cells. NK cell migration towards CXCL10/11 was associated with significant upregulation of CXCR3 upon IL-15+IL-18 stimulation for PB NK cells (80.15 ± 2.67 %) and CB NK cells (89.98 ± 0.69 %) ($p = 0.012$) (Fig 5.9 D).

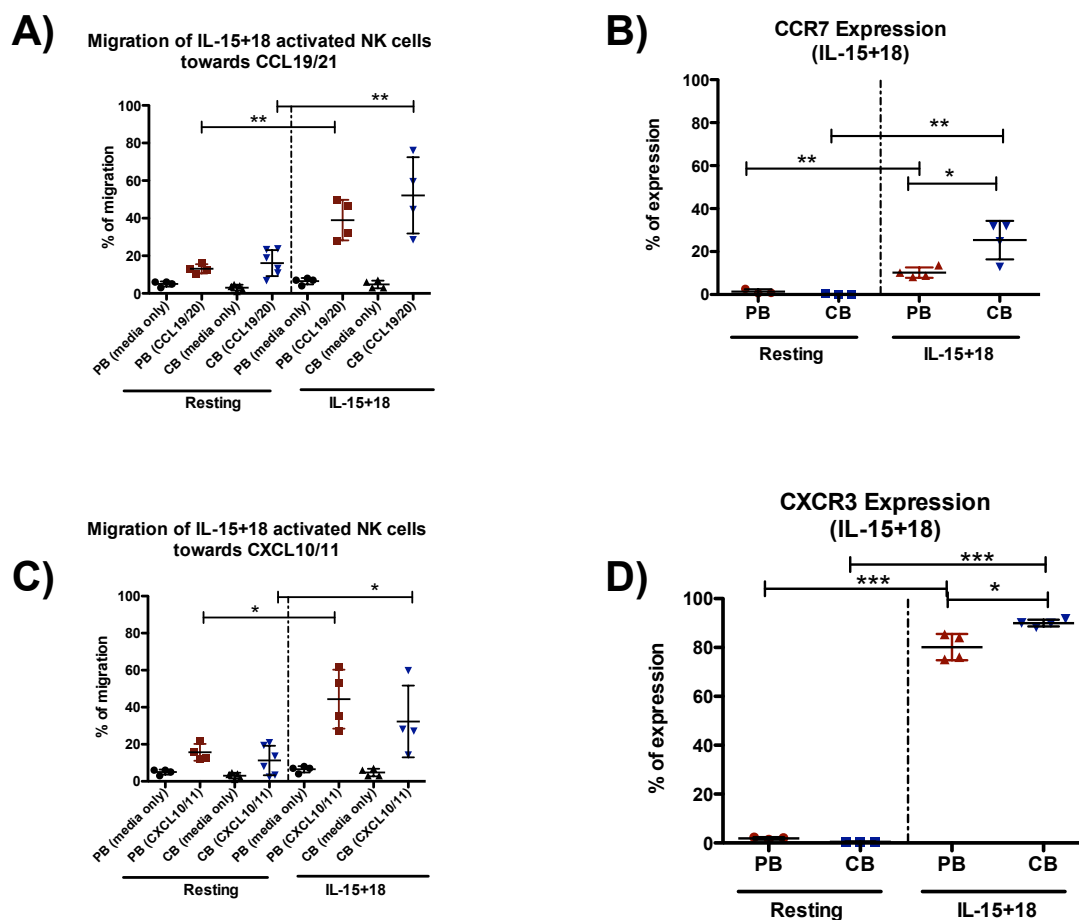


Figure 5.9 Migration of IL-15+IL-18 activated PB and CB NK cells in response to lymph node chemoattractants. A) Percentage of migration of PB and CB NK cells towards CCL19/CCL21 by both resting and IL-15+IL-18 activated NK cells. B) CCR7 expression by NK cells activated with IL-15+IL-18, as compared to resting NK cells. C) Percentage of migration of PB and CB NK cells towards CXCL10/11 by both resting and IL-15+IL-18 activated NK cells. D) CXCR3 expression by NK cells activated with IL-15+IL-18, as compared to resting NK cells. This analysis was performed on purified NK cells 2 days post IL-15+IL-18 stimulation. Migrated cells were collected from the lower chamber of the trans-well plate and the percentage of migration was calculated as $(\text{no of migrated cells} \div \text{positive control}) \times 100$. Results are presented as Mean \pm SD. N=4, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

5.3 Discussion

To exert their effector functions, NK cells need to express receptors that allow their recruitment to sites of inflammation (Robertson et al., 2000). Notably, as for other lymphocytes, the regulation of NK cell trafficking is chemokine-dependent. Resting CB NK cells have been shown to have low expression of L-selectin, which may influence their homing to draining LN. However, it was confirmed here that cytokine activation induces the expression of L-selectin and other chemokine receptors resulting in migration towards their cognate ligands *in vitro*.

L-selectin is an adhesion molecule that plays a key role in initiating extravasation of lymphocytes towards the luminal surface of HEV. PB CD56^{dim} NK cells express very low levels of L-selectin, whereas CD56^{bright} NK cells express a high density of L-selectin (Nagler et al., 1989, Sedlmayr et al., 1996). It has been reported that L-selectin is upregulated by both CD56^{bright} and CD56^{dim} PB NK cells in response to IL-12 (Frey et al., 1998). In contrast, it has been found that IL-2 and IL-15 downregulate L-selectin expression by PB NK cells (Frey et al., 1998, Uksila et al., 1997, Mäenpää et al., 1993). The data presented in this chapter are in line with these reports and suggest that PB NK cells may experience rapid loss of L-selectin expression after they reach peak activation with IL-2 and IL-15 (Frey et al., 1998). However, neither CD56^{bright} nor CD56^{dim} NK cells express L-selectin in CB, which might affect their trafficking capability (Dalle et al., 2005, Luevano et al., 2012a). Remarkably, incubation with IL-12 or IL-18 induced L-selectin expression on CB NK cells to a level comparable to PB NK cells. This suggests that the induction of L-selectin expression by NK cells may require exposure to certain cytokines, such as IL-12 or IL-18, produced by activated DCs or macrophages. Indeed, variable expression of L-selectin by PB CD56^{dim} NK cells has been shown in many reports (Frey et al., 1998, Mäenpää et al., 1993, Uksila et al., 1997) and was confirmed here. Interestingly, it has previously been reported that CD56^{dim} NK cells acquire phenotypic characteristics of less mature NK cells in response to infection and that they express a high density of L-selectin (Frey et al., 1998).

Only CD56^{bright} PB NK cells have the capacity to traffic to LN. Their migratory capacity to LN correlates with the expression of some chemokine receptors including

CCR7 and CXCR3 (Campbell et al., 2001). It has been reported that CCR7 expression could be induced on CD56^{dim} NK cells *in vitro* by incubation with IL-18 (Mailliard et al., 2005). Furthermore, it has been reported that KIR⁺ NK cells are capable of CCR7 uptake from mature DC and can therefore migrate to LN where they bind to their inflammatory chemokine ligands, CXCL10/CXCL11 and/or CCL19/CCL21. NK cell recruitment to the LN is important during haplo-identical HSCT, as alloreactive NK cells can kill host DCs that are responsible for priming donor T cells and subsequently reduce GvHD (Marcenaro et al., 2009). In mice, NK cell recruitment to lymphoid tissues is dependent on CXCR3 expression due to the ability of this receptor to bind to CCL21 (Martín-Fontecha et al., 2004). NK cell trafficking to draining LN in response to CCL19/21 in humans is CCR7-dependent (Campbell et al., 2001, Kim et al., 1999). However, it is noteworthy that mechanisms underlying NK cell homing to LN are not fully understood, as blocking L-selectin expression has been shown to significantly reduce NK cell recruitment to LN (Martín-Fontecha et al., 2004). Hence, it is conceivable that NK cells use one or more chemokine receptors in order to traffic to LN. CB NK cells treated with cytokines upregulated CXCR3 in all conditions tested. However, inconsistencies were observed regarding CCR7 expression using two different monoclonal antibodies, which could be due to the fact that these antibodies recognise different epitopes.

In the current study, I showed that the percentage of resting NK cells migrating in response to CCL19/21 was low (less than 20 %). However, stimulation with cytokines enhanced the migration of NK cells, and this was associated with an upregulation of CCR7. CCL19 and CCL21 are differentially distributed within the LN but both are ligands for CCR7, whereas CCL19 is expressed by DC residing in the T cell zone of the LN (Ngo et al., 1998), CCL21 is expressed by lymphatic endothelial cells in the T cell zone of the LN (Willmann et al., 1998). NK cell trafficking to LN allows interaction with LN resident cells including DC and T cells, to result in a potent production of IFN- γ (Scharton and Scott, 1993). In addition, CCL19/21 has been shown to co-stimulate the proliferation of IL-2 activated CD56^{dim} NK cells (Robertson et al., 2000). Hence, CCR7-mediated trafficking of NK cells towards CCL19/21 may confer the ability of NK cells to migrate to LN, where many tumour types tend to metastasise (Zlotnik, 2006). Herein, I showed enhanced migration towards CCL19/21 by PB and CB NK cells activated with IL-15+IL-2 or IL-15+IL-18. Both IL-15 and IL-2 have been

reported to induce chemotaxis of NK cells facilitating their attachment to endovascular epithelium in an LFA-1-dependent manner (Allavena et al., 1997). Yet, IL-18 appeared to be a major factor in NK cell recruitment to LN by increasing CCR7 expression by NK cells.

Trans-well migration assays were performed to assess NK cell migratory capacity *in vitro* towards CXCL10/11. Herein, the percentage of migration by resting NK cells towards CXCL10/11 was less than 20 %. Upon cytokine activation, CXCR3 expression was substantially upregulated by PB and CB NK cells. As a result, cytokine-activated NK cells showed enhanced migration towards CXCR3 ligands CXCL10/11. The LN does not typically express CXCL10/11. In fact, CXCL10/11 is an inducible chemokine ligand that is expressed by infected macrophages and DCs in response to IFNs type I and II (Cole et al., 1998). The expression of CXCL10/11 leads to recruitment of CXCR3⁺ NK cells to their target cells. However, it has been shown that CXCL10/11 not only recruits effector cells to target cells, but also that it is involved in migration and the retention of T cells into the draining LN in HIV patients (Foley et al., 2005). Moreover, the expression of CXCL10/11 was induced in the LN of rats infected with autoimmune encephalomyelitis (McColl et al., 2004). Although it has been shown that mouse NK cells expressing CXCR3 could bind CCL19/21 in LN, it is also possible that CXCL10/11 is produced by follicular DCs present in LN stroma. The movement of activated NK cells from the red pulp to the white pulp of the spleen, which is the T cell zone, was found to be CXCR3-dependent in a MCMV mouse model (Grégoire et al., 2008). Hence, CXCR3 expression is vital for recruitment of NK cells to both inflamed non-lymphoid sites and to LN. It was found here that NK cells activated with IL-15+IL-2 or IL-15+IL-18 showed higher migration than those activated with IL-15+IL-12. This was associated with less expression of CXCR3 upon IL-15+IL-12 stimulation compared to IL-15+IL-2 or IL-15+IL-18 stimulation. Interestingly, it was reported that IL-12, alone or in combination with IL-2, rapidly downregulates CXCR3 mRNA expression by NK cells affecting their trafficking towards CXCL10. This was suggestive of an immunomodulatory role for NK cells during inflammation whereby NK cells could downregulate the expression of certain chemokine receptors to skew the immune response towards a more T helper cell type response when acquired (Hodge et al., 2002). However, it remains unknown why NK cells exhibit less migration following stimulation with IL-15+IL-12 in comparison to other conditions.

To summarise, PB and CB NK cells upregulate receptors involved in migration to the LN following cytokine stimulation including L-selectin, CCR7 and CXCR3. Particularly, IL-12 and IL-18 were shown to be potent inducers of L-selectin expression on CB NK cells. Combinations of IL-15+IL-2 or IL-15+IL-18 were shown to promote *in vitro* migration of PB and CB NK cells in response to CCL19/21 or CXCL10/11, which correlated with upregulation of the corresponding chemokine receptors by NK cells.

Chapter 6: Cytokine-induced memory-like NK cells

6.1 Introduction

Immunological memory is defined based on specific antigen recognition and a robust recall response upon subsequent exposure to the same antigen. NK cells are designated as innate immune cells due their ability to recognise target with no prior sensitisation and to exert cytotoxic functions accordingly. As innate immune cells, it was thought that they respond to a stimulus in a similar manner regardless of repeated exposure. Surprisingly, NK cell immunological memory has been described in MCMV infected mice (Sun et al., 2009). In this model, Ly49H⁺ NK cells that were transferred into Ly49H⁻ mice experienced antigen-specific expansion following MCMV infection. Furthermore, these long-lived cells underwent robust secondary expansion, and exhibited enhanced cytotoxicity and protection against MCMV infection.

In addition, it was shown by Yokoyama and colleagues that cytokine pre-activation could induce memory-like NK cells in mice (Cooper et al., 2009). In this model, IL-12 and IL-18 pre-activated NK cells infused into naïve hosts could still be detected 22 days post-infusion. Moreover, when these cells were restimulated with IL-15 and IL-12 they displayed high production of IFN- γ . Subsequent studies have also showed similar findings with regard to high IFN- γ production by cytokine pre-activated human NK cells (Romee et al., 2012).

Herein, I analysed whether cytokine pre-activation of CB NK cells would induce long-lived NK cells with memory-like features, as has been observed for PB memory-like NK cells with respect to IFN- γ production. Hence, I followed the published protocol of Romee and colleagues to investigate whether memory-like NK cells can be generated from CB using cytokines (Romee et al., 2012), using PB NK cells as a control.

6.2 Results

The following analysis focused on IFN- γ production, secretion and gene expression by memory-like NK cells generated from PB and CB. The study was carried out using NK cells that were pre-activated with IL-15 only (control NK cells) or NK cells that were pre-activated with IL-12, IL-15 and IL-18 (pre-activated NK cells). IFN- γ analysis was performed after restimulation of NK cells with IL-12 and IL-15 at different time points for up to 3 weeks.

6.2.1 Generation of a higher frequency of IFN- γ producing PB and CB NK cells following pre-activation with cytokines

The production of IFN- γ by cytokine-induced memory-like NK cells from PB and CB was investigated by flow cytometry at days 7, 14 and 21 of culture by intracellular staining. Overall, levels of intracellular IFN- γ were more variable for control NK cells than for pre-activated NK cells. This pattern was identical for cells generated from both PB (Fig 6.1) and CB (Fig 6.2). At day 7, the percentage of IFN- γ producing PB NK cells was 43.48 ± 2.71 % for control NK cells and 71.08 ± 2.72 % for pre-activated NK cells ($p < 0.0001$). Similarly, 40.02 ± 3.78 % and 60.02 ± 1.48 % of control NK cells and pre-activated NK cells from CB produced IFN- γ ($p = 0.0006$) (Fig 6.3). At day 14, the mean percentage of IFN- γ production was 48.96 ± 4.04 % and 43.50 ± 3.28 % for PB and CB control NK cells respectively (Fig 6.3). Similarly to day 7, IFN- γ production was significantly increased at day 14 for pre-activated NK cells from PB (64.60 ± 0.927 %) and CB (53.33 ± 1.49 %) as compared to control NK cells ($p = 0.0055$ and 0.0214 respectively). At day 21, a comparable pattern of IFN- γ expression was observed for pre-activated NK cells from PB (55.86 ± 1.86 %) and CB (52.95 ± 3.30 %) than for control NK cells from PB (40.14 ± 2.99 %) and CB (39.00 ± 3.93 %) ($p = 0.0021$ and 0.0217 respectively) (Fig 6.3). Figure 6.4 shows the MFI of IFN- γ expression as compared to control and pre-activated NK cells. Cytokine-induced memory-like NK cells were checked for viability at day 21 using 7AAD only (Fig 6.5). Therefore, further analysis is required to examine their apoptotic state using both Annexin V and 7AAD through out different time points.

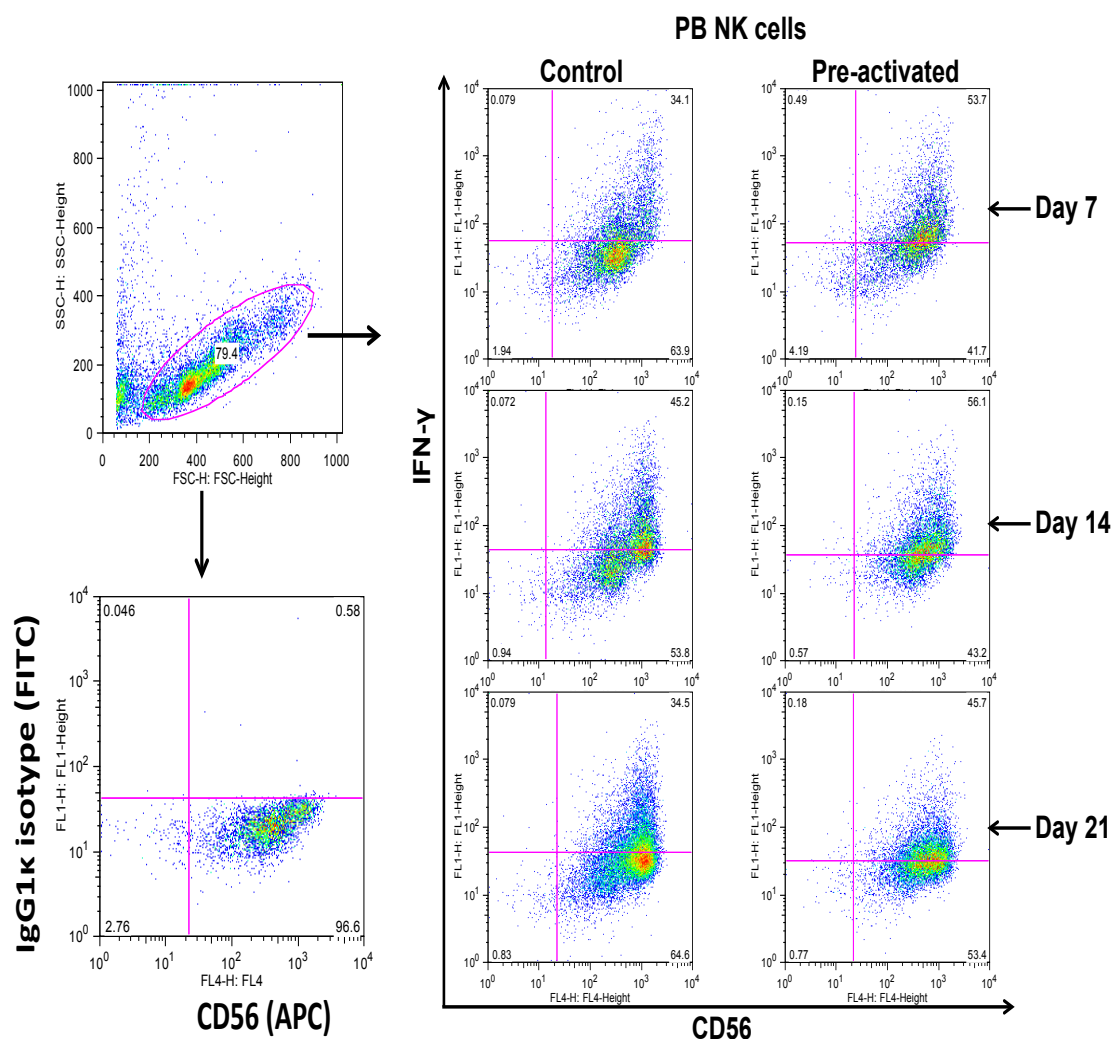


Figure 6.1 Flow cytometric histograms showing IFN- γ production by cytokine-induced memory-like NK cells derived from PB NK cells. The graphs compare IFN- γ production at days 7, 14 and 21 for control (unstimulated) NK cells (left panel) and pre-activated (stimulated with IL-12 and IL-18) NK cells (right panel) from one representative PB sample. Analysis was performed 6 hr after restimulation with IL-12 and IL-15. Cut-off values were adjusted based on negative control isotype.

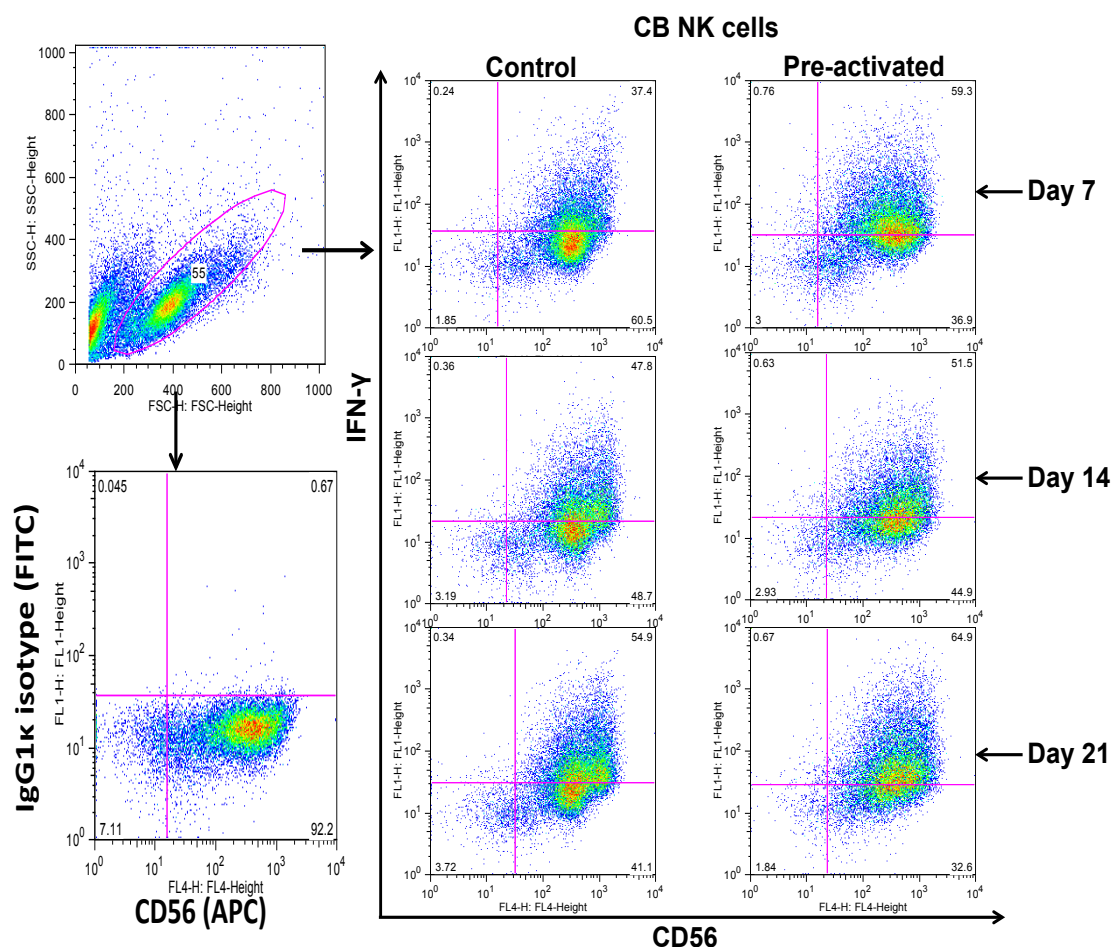


Figure 6.2 Flow cytometric histograms showing IFN- γ production by cytokine-induced memory-like NK cells derived from CB NK cells. The graphs compare IFN- γ production at days 7, 14 and 21 for control (unstimulated) NK cells (left panel) and pre-activated (stimulated with IL-12 and IL-18) NK cells (right panel) from one representative CB sample. Analysis was performed 6 hr after restimulation with IL-12 and IL-15. Cut-off values were adjusted based using an isotype.

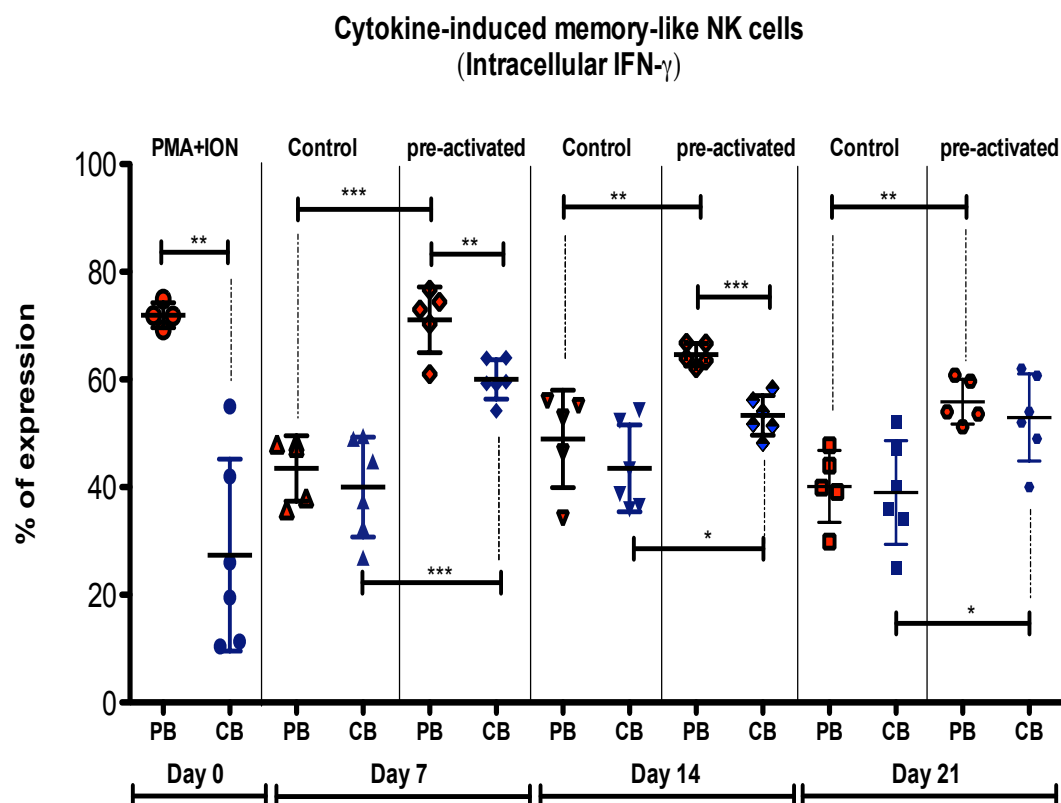


Figure 6.3 Comparison of IFN- γ production by cytokine-induced memory-like NK cells derived from PB and CB NK cells. The graph represents intracellular expression of IFN- γ analysed by flow cytometry at days 7, 14 and 21 in control and pre-activated NK cells from PB (red symbols) and CB (blue symbols). PMA and Ionomycin were used as positive control for induction of IFN- γ expression (at day 0 only as cells would die if left with no cytokines). Analysis was performed 6 hr after restimulation with IL-12 and IL-15. Results are presented as Mean \pm SD. N=5 for PB and 6 for CB, p < 0.01 (**), and p < 0.001 (***).

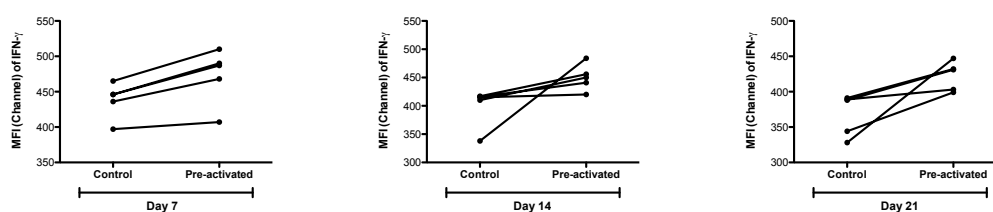
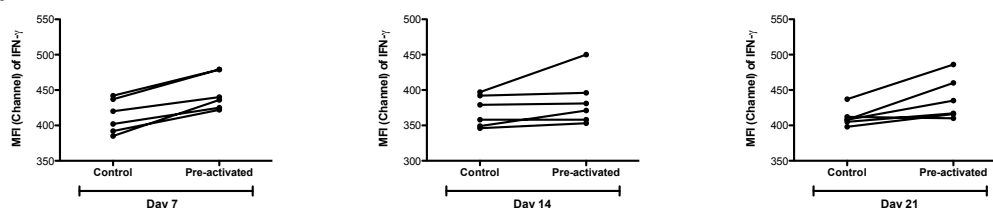
A) PB memory-like NK cells**B) CB memory-like NK cells**

Figure 6.4 Mean fluorescence intensity of intracellular IFN- γ by cytokine-induced memory-like NK cells generated from PB (A) and CB (B). This figure compares MFI of IFN- γ expression between controls and pre-activated NK cells at days 7, 14 and 21. N=5 for PB and 6 for CB.

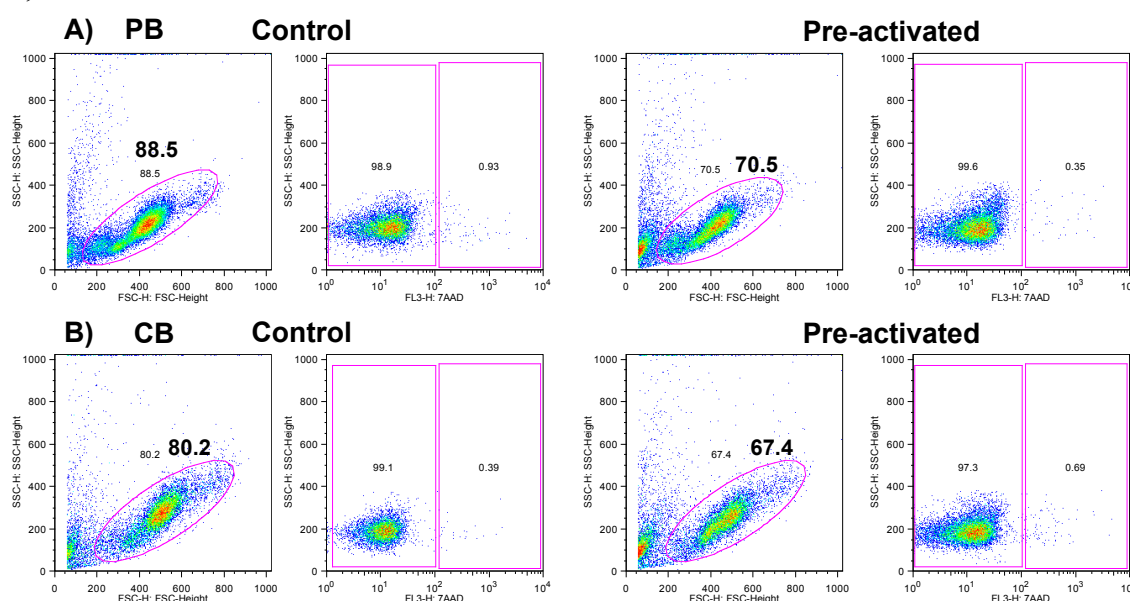


Figure 6.5 Cytokine-induced memory-like NK cell viability. The viability of cytokine-induced memory-like NK cells generated from PB (A) and from CB (B) tested using 7AAD at day 21.

6.2.2 IFN- γ secretion by memory-like NK cells does not correlate with the frequency of IFN- γ^+ expressing NK cells

Culture supernatants from cytokine-induced memory-like NK cells were collected and tested for IFN- γ secretion using multiplex bead-based immunoassays. IFN- γ secretion by PB NK cells was higher than for CB NK cells in both control and pre-activated NK

cell samples (Fig 6.6). At day 7, IFN- γ secretion by control and pre-activated PB NK cells was not significantly different (3950 ± 862.0 pg/mL and 5109 ± 794.6 pg/mL respectively). In contrast to PB, pre-activated CB NK cells secreted significantly higher IFN- γ (1586 ± 272.2 pg/mL) than control CB NK cells (153.4 ± 44.55 pg/mL) ($p = 0.0020$). At day 21, IFN- γ secretion by control PB NK cells was surprisingly higher than pre-activated PB NK cells (5109 ± 794.6 pg/mL and 1518 ± 435.2 pg/mL respectively, $p = 0.0042$), suggesting that cytokine pre-activation of PB NK cells may induce exhaustion in long-term cultures. Control CB NK cells secreted comparable levels of IFN- γ to pre-activated CB NK cells (1035 ± 226.1 pg/mL and 912.5 ± 194.9 pg/mL respectively) (Fig 6.6). Notably, memory-like NK cells did not secrete any of the other cytokines tested by multiplex including TNF- α , TNF- β , IL-6 or IL-8.

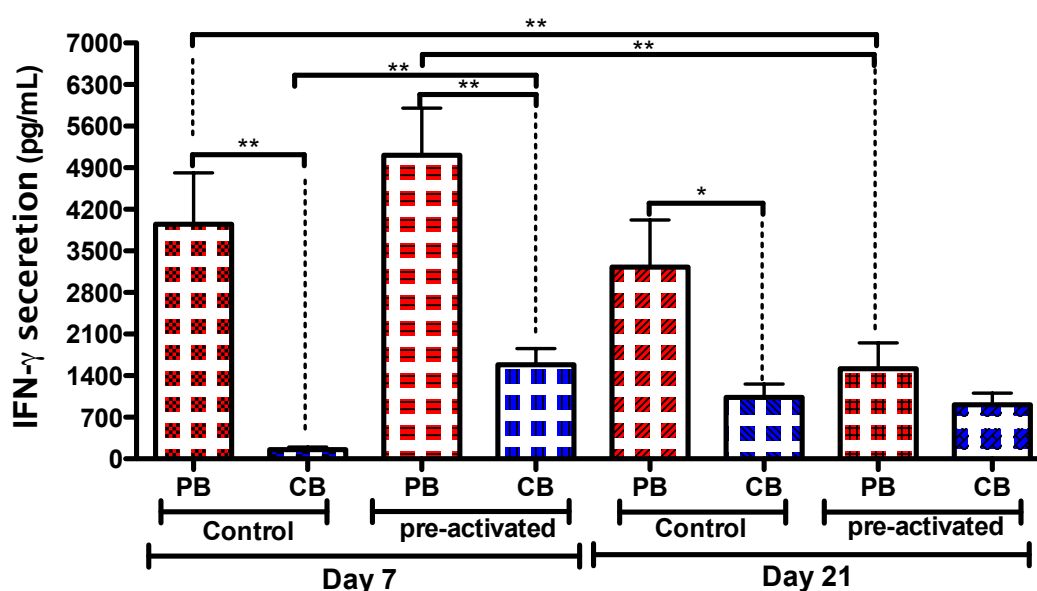


Figure 6.6 IFN- γ secretion by cytokine-induced memory-like NK cells. Secretion of IFN- γ by cytokine-induced memory-like NK cells derived from PB (red bars) and CB (blue bars) NK cells at days 7 and 21 of culture measured by multiplex bead-based immunoassay. Supernatants were collected 6 hr after restimulation with IL-12 and IL-15. Results are presented as Mean \pm SD. N=5 for PB and 4 for CB, $p < 0.05$ (*) and $p < 0.01$ (**).

6.2.3 Equivalent gene expression of IFN- γ by control and pre-activated NK cells from PB and CB

IFN- γ gene expression was analysed by real-time PCR in comparison to reference genes (Fig 6.7). Equivalent relative expression of IFN- γ was detected for control and pre-activated NK cells from PB (0.9703 ± 0.034 and 0.9937 ± 0.021 respectively) and CB (0.9155 ± 0.005 and 0.9088 ± 0.012 respectively). Interestingly, pre-activated CB NK cells exhibited significantly higher IFN- γ gene expression than pre-activated PB NK cells ($p = 0.0057$).

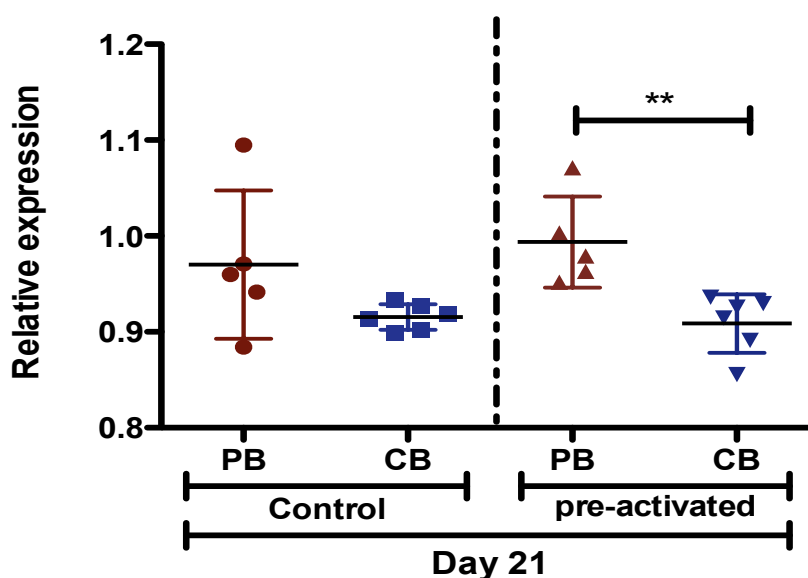


Figure 6.7 IFN- γ gene expression by cytokine-induced memory-like NK cells. Real-time PCR analysis of IFN- γ mRNA levels in control and pre-activated NK cells from PB (red symbols) and CB (blue symbols). Gene expression was normalised using three reference genes (ACTB, β 2M and UBC). Results are presented as Mean \pm SD. N=5 for PB and 6 for CB, $p < 0.01$ (**).

6.3 Discussion

It was recently shown that NK cells develop features of immunological memory under certain conditions including, exposure to infection, sensitisation with specific antigens such as haptens, or pre-activation with cytokines (Min-Oo et al., 2013). It has been shown that MCMV-induced-memory NK cells undergo secondary expansion as well as enhanced cytotoxicity and specificity to m175 viral proteins following re-exposure to antigen (Sun et al., 2009). Similarly, liver-restricted-memory NK cells have been shown to exhibit cytotoxicity and specificity to the sensitising antigen, but it remained to be determined whether these cells could undergo secondary expansion (O'Leary et al., 2006, Paust et al., 2010). On the other hand, cytokine-induced memory-like NK cells have not been shown to exhibit any change in their cytotoxic function, rather, they tend to produce higher levels of IFN- γ (Cooper et al., 2009). Although memory-like NK cells generated from different sources exhibited different properties in regards to their cytotoxic functions, they share a common feature of immunological memory, which is extended lifespan. Hence, the aim of this chapter was to generate long-lived memory-like NK cells from PB and CB NK cells using cytokines, following the published protocol of Romee and colleagues (Romee et al., 2012), and to address whether CB NK cells produce IFN- γ in a similar manner to PB NK cells following cytokine stimulation.

My results show that restimulation of pre-activated PB and CB NK cells with IL-12 and IL-15 results in a higher frequency of IFN- γ producing cells than for control NK cell cultures. NK cells are known to be short-lived *in vitro* (5 to 7 days) (Cooper et al., 2002) and NK cells require high doses of cytokines in order to maintain their viability in culture. Herein, NK cells were maintained *in vitro* with a very low dose of IL-15 for up to 3 weeks. The fact that NK cells are long-lived in culture following cytokine stimulation corresponds to one of the properties of memory NK cells.

In addition, different patterns of intracellular IFN- γ production were observed between control and pre-activated NK cells from PB and CB. It was evident that the vast majority of pre-activated NK cells produce IFN- γ , whereas only a subset of control NK cells did. In control NK cell cultures, the main producers of IFN- γ are the CD56^{bright} population. This is in contrast to pre-activated NK cell cultures whereby all NK cells

produce IFN- γ . In accordance with previous studies, cytokine pre-activation increases the frequency of IFN- γ producing NK cells (Cooper et al., 2009, Romee et al., 2012). The finding that NK cells become IFN- γ producing cells upon secondary stimulation indicates that NK cells preserve memory to previous cytokine exposure and exhibit a robust response when re-exposed to the same cytokine stimulation. However, this memory property is not specific to a single cytokine such as IL-12, IL-15 or IL-18 which are not capable to induce similar properties alone, whereas pre-activation with combinations of IL-12+IL-18, IL-15+IL-18 or IL-15+IL-12 are sufficient to induce memory imprint of NK cells (Romee et al., 2012). Interestingly, it was shown that this pattern of IFN- γ production is exhibited for both cells that have proliferated and non-proliferated cells (Cooper et al., 2009). Hence, it is likely that this feature of memory-like NK cells is passed onto successive generations of cells, although this was not investigated here.

CB NK cells are considered to be naïve cells due to their immature phenotype and decreased cytotoxic function as compared to PB NK cells (Gaddy et al., 1995, Luevano et al., 2012a). Moreover, stimulation of CB NK cells with mitogens such as PMA and Ionomycin resulted in significantly less IFN- γ production compared to PB NK cells. Nevertheless, it was possible to promote IFN- γ production by CB NK cells by pre-activation with cytokines. Hence, it could be that the exposure of CB NK cells to inflammatory cytokines induces intrinsic modification of CB NK cells, and that cytokine pre-activation modifies the cytokine receptor repertoire and the responsiveness to cytokine activation accordingly. In this context, it was shown that pre-activation of NK cells with IL-12 and IL-18 increased IL-2R α expression (Ni et al., 2012). As for cytokine-induced memory-like PB NK cells, mechanisms underlying these changes remain to be clarified.

Although the vast majority of pre-activated NK cells were IFN- γ producing cells, IFN- γ secretion was variable among control and pre-activated NK cells, as well as between different sources of NK cells. I showed here that restimulation of pre-activated PB NK cells induces higher secretion of IFN- γ than for control NK cells at day 7. However, IFN- γ secretion by PB memory-like NK cells at day 21 was not consistent with intracellular IFN- γ production, as control NK cells secreted higher IFN- γ than pre-activated NK cells. Control CB NK cells at day 7 secreted significantly less IFN- γ than

pre-activated CB NK cells. By day 21, it appeared that control and pre-activated CB NK cells secreted similar amounts of IFN- γ . In general, memory-like NK cells generated from CB NK cells secreted lower levels of IFN- γ than those generated from PB NK cells. It is noteworthy that previous studies that aimed to generate memory-like NK cells using cytokines have detected intracellular expression of IFN- γ in memory-like NK cells, rather than IFN- γ secretion (Cooper et al., 2009, Romee et al., 2012). Hence, my findings suggest that although pre-activated NK cells display higher intracellular IFN- γ , it does not necessarily correlate with IFN- γ secretion *in vitro*. It remains to be clarified whether these cells would behave similarly *in vivo*. Furthermore, there is the possibility that exposure to cytokines resulted in exhaustion of NK cells as control NK cells appeared to secrete higher levels of IFN- γ than pre-activated NK cells.

Finally, I investigated IFN- γ mRNA levels to assess whether IFN- γ gene expression differs between control and pre-activated groups of memory-like NK cells. In accordance with other reports, my results showed that there is no difference in IFN- γ gene expression between the two groups, for both PB and CB NK cells. However, I showed here that CB NK cells in general express higher levels of IFN- γ than PB NK cells. The fact that comparable IFN- γ gene expression was seen in control and pre-activated NK cells confirms that the higher production of IFN- γ is not the result of an enhanced transcription of IFN- γ . Rather, memory-like NK cells may experience post-transcriptional or post-translational modifications to regulated IFN- γ secretion, possibly occurring at other loci than the *ifng* locus (Romee et al., 2012, Stetson et al., 2003).

To summarise, I generated long-lived NK cells *in vitro* using cytokines from PB and from CB NK cells for the first time. Cytokine pre-activated NK cells showed higher IFN- γ production than the control NK cells upon restimulation with IL-12 and IL-15. However, this did not correlate with higher secretion of IFN- γ or to higher IFN- γ mRNA expression. Other aspects of cytokine-induced memory-like NK cells remain to be investigated to understand the underlying mechanisms of these interesting findings.

Chapter 7: Conclusions and future directions

7.3 Background of the current study

NK cells are key players in the eradication of virally infected cells and transformed cells and have therefore been considered as a cell therapy option for cancers. Different groups have explored the use of cytokines to either expand NK cells or to enhance their cytotoxicity using NK cells from PB or CB. Unlike PB NK cells, freshly isolated CB NK cells are unable to mediate cytotoxic activities, however, they have been shown to be cytotoxic after cytokine stimulation. Studying the optimal activation conditions for different sources of NK cells will provide fundamental information for future NK cell-based therapies. Hence, the aim of this study was to induce fully functional CB NK cells using cytokines and to perform comparative analysis of the mechanisms of activation between PB and CB NK cells. Herein, I was able to identify differential responsiveness of CB and PB NK cells to cytokine activation.

7.4 Summary of key results

Table 7.1 summarises my findings in regard to NK cell activation by cytokines. I observed that IL-2 promoted optimal effector functions of PB NK cells (upregulation of activating receptors, killing capacity and cytokine production), whereas CB NK cells did not respond similarly to IL-2 stimulation even when higher doses were used. Moreover, I found that IL-15 or IL-18 stimulation induced upregulation of activating receptors on both PB and CB NK cells. However, KIR and CD94/NKG2A expression was not altered by cytokine stimulation whereby cytokine-activated CB NK cells still exhibited low KIR expression and high CD94/NKG2A expression.

All cytokine stimulation conditions used enhanced killing of K562 cells by PB NK cells after two days of stimulation with cytokine. However, the killing capacity of CB NK cells was the highest post-stimulation with IL-15 or IL-15+IL-2, whereas IL-18 stimulation had no effect. After 5 days of stimulation, the killing capacity of CB NK cells was enhanced after IL-15+IL-2 stimulation, showing higher killing levels than PB NK cells. I confirmed that PB and CB NK cells have equivalent mRNA levels of

perforin regardless of the cytokine stimulation used whereas the expression of granzyme B was variable among different cytokine stimulation strategies. In regard to cytokine production, PB NK cells secreted high amounts of IFN- γ post-stimulation with IL-2, whereas CB NK cells secreted the highest levels of IFN- γ and TNF- α after stimulation with the combination of IL-15+IL-18 (Table 7.1).

Table 7.1 Summary of NK cell activation study

	IL-2		IL-12		IL-15		IL-18		IL-15+IL-2		IL-15+IL-18	
	PB NK cells	CB NK cells	PB NK cells	CB NK cells	PB NK cells	CB NK cells	PB NK cells	CB NK cells	PB NK cells	CB NK cells	PB NK cells	CB NK cells
Activating receptors	+++ (*)	+	++	+++	+++	+++	+++	+++	NI	NI	NI	NI
KIR expression	CB NK cells express less KIR than PB NK cells and cytokine stimulation has no impact on KIR expression											
CD94/NKG2A	CB NK cells express higher levels of CD94/NKG2A than PB NK cells and cytokine stimulation has no impact on CD94/NKG2A expression											
Proliferation	++	+	D2 Only (+)	D2 Only (+)	+	++	D2 Only (+)	D2 Only (+)	+	++	+++	+++
K562 killing at D2	+++	+	+++	++	+++	+++	++	–	++	++	+++	+
K562 killing at D5	++	++	NI (**)	NI	+++	++	NI	NI	++	+++	++	++
IFN-γ gene expression (***)	Upregulated	Similar to resting	Similar to resting	Upregulated	Upregulated	Similar to resting	Upregulated	Similar to resting	Upregulated	Similar to resting	Upregulated	Upregulated
IFN-γ secretion (D2)	++	+	+	+++	++	++	+	+	+	+	++	+++
IFN-γ secretion (D5)	+++	+	NI	NI	++	++	NI	NI	+	+	++	+++
TNF-α secretion (D2)	+	+	–	–	++	++	–	+++	+	+	+	+++
TNF-α secretion (D5)	+	+	NI	NI	+	+	NI	NI	+	+	+	++
L-selectin expression	+++	–	+++	+++	++	–	+++	++	NI	NI	NI	NI
CXCR3 expression	++	+++	+	++	+++	+++	++	++	+++	+++	++	+++
CCR7 expression (3D12/358426 clones)	+/+	–/+	–/+	+/+	+/+	+/+	–/+	–/+	+	++	+	++
Migration in response to CXCL10/11	NI	NI	NI	NI	NI	NI	NI	NI	+++	+++	++	++
Migration in response to CCL19/21	NI	NI	NI	NI	NI	NI	NI	NI	+++	+++	++	++

(*) = +++ > ++ > + > –, (**) = NI (not identified), (***) = Gene expression evaluated as upregulated or downregulated compared to resting NK cells.

7.3 Caveats and weakness of the study

Although this study presents differential findings in regards to activation of PB and CB NK cells, there is variability to consider between the two sources. PB samples were collected within the department and processed freshly after collection whereas CB samples were collected at different hospitals and transported to the Anthony Nolan Therapy Centre, Nottingham, UK. Thereafter, CB samples were dispatched to the Anthony Nolan Research Centre, London, UK. Therefore, the time between CB collection and processing may vary among samples leaving a time gap of 24 hrs to 48 hrs between CB collections and processing that may affect CB viability. Hence, the time course could contribute to alteration of NK cell characteristics between the two sources including phenotype and effector functions (Naranbhai et al., 2011).

One main weakness of the study was the inability to perform analysis on different subsets of NK cells; CD56^{bright} and CD56^{dim} after cytokine stimulation. This was due to the alteration of CD56 expression on NK cells post stimulation that becomes more homogeneous rendering difficult separation between the two subsets. Another limitation factor to consider in this study is sample number. The analysis was performed on 4 to 5 samples for each condition due to time constraints. Although significant findings were observed, sample size remains an important factor to detect genuine variation between PB and CB NK cells and to ensure reproducibility of each experiment. Hence, future analysis necessitates increasing of the sample size for all analysis performed.

In addition, a cytokine dose-dependent responsiveness study is yet to be performed. In spite that CB NK cells showed maximal activation using lower doses of IL-15 (20 ng/mL) and IL-12 (10 ng/mL), it is possible that lower doses may induce similar effects on CB NK cells. Therefore, future analysis may aim to optimise cytokine concentrations by performing activation study with lower doses of cytokines. Also, the IL-12, IL-15 and IL-18 concentration remained to be adjusted to an international unit (IU) instead of grams to provide more consistent biological activity of these cytokines.

As for flow cytometric analysis, unstained cells were used as negative controls (except for signaling and intracellular IFN- γ studies where isotype has been included). Therefore, suitable non-reactive isotype for each monoclonal antibody should have

been included in the phenotype study. Also, the inclusion of fluorescence minus one (FMO) control which include all fluorochromes except the one being tested, would offer the identification of the spread of any other fluorochromes into the channel of interest, and hence proper separation between negative and positive population could be achieved.

In addition, the expression of cytotoxic mediators was assessed by real time PCR to measure mRNA levels of granzyme B and perforin. This was done to detect any change that could occur at the gene expression post cytokine stimulation and to perform comparison between cytokine-activated PB and CB NK cells. However, my results showed no significant findings that could be the result of transcription in order to assemble granzyme B and perforin proteins. Hence, mRNA levels may not be an actual indicative for NK cell cytotoxicity. Future analysis should be performed to assess the intracellular expression of granzyme B and perforin instead of gene expression to be able to detect possible variation between the two sources post cytokine stimulation as well as a comparison with resting NK cells.

Cytokine secretion by activated NK cells was performed by Multiplex (bead-based immunoassay), which offered the detection of secretion of many cytokines in this study. However, those analyses were performed on the supernatant of cytokine activated NK cells that have been purified using negative selection. Despite that the purity of isolated NK cells was exceeding 90 %, there could be other possible contaminants produced by other cells remained after isolation. Future analysis are still required by using sorted CD56⁺ NK cells in similar experiments to confirm my findings.

7.4 Physiological relevance of this study

Human neonates have a naïve adaptive immune system and are more susceptible to infections than adults, in particular preterm infants (Berrington et al., 2011). In this context, infants are not able to develop T_H1 responses against bacterial and viral infections. In contrast, T cell responses are shown to be skewed towards T_H2 responses, which mediate a suppressive role, and hence limiting inflammation and tissue damages in infants (Zaghouani et al., 2009). It has been suggested recently that IL-8 plays a major role in neonatal immunity as increased IL-8 production was detected by neonatal CD4⁺ T cells as compared to adult CD4⁺ T cells after stimulation with PMA and

Ionomycin or anti-CD3+CD28 beads (Gibbons et al., 2014). In addition, IL-8 has been shown to co-stimulate the production of IFN- γ from neonatal $\gamma\delta$ T cells (Gibbons et al., 2009).

IL-8 is a potent chemotactic factor of neutrophils mediating their migration towards infection sites where they exert their effector functions (Zlotnik and Yoshie, 2012). Herein, phenotypical and functional differences between adult PB and CB NK cells observed in this study may correlate with different physiological roles mediated by neonatal NK cells than those in adults. Similar to neonatal T cells, CB NK cells produced substantial amounts of IL-8 post cytokine stimulation as compared to adult NK cells, which may provide protection against neonatal infections by enhancing migration of neutrophils to infected sites to provide neutrophil-mediated anti-microbial response. Also, this finding highlights the necessity to further investigate the intracellular expression of IL-8 by CB NK cells to be able to quantify the percentage of IL-8-producing NK cells in CB and to gain insight to the role of neonatal NK cells in infants.

7.5 Is interleukin-2 the “ideal” cytokine to stimulate CB NK cells?

It has been previously reported that CB NK cells require up to 10 times more IL-2 than PB NK cells for activation and expansion (Condiotti et al., 2001, Luevano et al., 2012a). Herein, I showed that CB NK cells express less IL-2R α than PB NK cells. In addition, STAT5 phosphorylation following IL-2 stimulation was significantly reduced in CB NK cells compared to PB NK cells. IL-2R α together with IL-2R β/γ mediates high affinity binding to IL-2, which promotes NK cell proliferation. In this context, CD56^{bright} NK cells express high levels of IL-2R α and therefore have enhanced proliferative capacities in response to IL-2, as compared to CD56^{dim} NK cells (Caligiuri et al., 1990, Carson et al., 1994, Cooper et al., 2001a). Accordingly, the reduced responsiveness of CB NK cells to IL-2 stimulation with respect to their expansion and cytotoxic functions could be a consequence of reduced high affinity binding to IL-2 given that CB NK cells express less IL-2R α and exhibit reduced phosphorylation of STAT5.

In addition, phosphorylation of STAT5 results in transcription of target genes such as IFN- γ . Phosphorylated STAT5 dimers bind to the *ifng* locus enhancing the production

of IFN- γ (Bream et al., 2004). I showed here that IL-2 activated CB NK cells secreted significantly less IFN- γ than IL-2 activated PB NK cells. Yet, I did not observe differential IFN- γ gene expression following IL-2 stimulation between PB and CB NK cells, although PB NK cells secreted higher levels of IFN- γ .

In the clinical context, IL-2 administration has been associated with toxicity, induction of AICD resulting in elimination of effector cells and expansion of Tregs (Ghiringhelli et al., 2005). *In vitro* studies showed that Tregs co-cultured with NK cells inhibit NK cell expansion by competing for IL-2 (Bachanova et al., 2014), as Tregs express IL-2R α (Yamaguchi and Sakaguchi, 2006). In contrast, parallel experiments have shown that the proliferation of IL-15 activated NK cells was not inhibited by Tregs as shown in (Bachanova et al., 2014). In the Bachanova study, successful expansion of donor NK cells along with systemic administration of IL-2 into AML patients was achieved by Treg cell depletion using an IL-2-diphtheria fusion protein. Interestingly, NK cell persistence and expansion was associated with clearance of AML cells and high serum levels of IL-15 indicating that IL-2-diphtheria fusion protein may indirectly enhance the production and presentation of endogenous IL-15 by DCs via the IL-15R α . This suggests that IL-15, but not IL-2, supplementation in NK cell-based therapies is essential for survival and persistence of adoptively transferred NK cells. Overall, I showed here that PB NK cells are optimally activated with IL-2, whereas IL-2 was not the ideal cytokine to activate CB NK cells. Thus, the use of IL-15 to activate CB NK cells should be taken into consideration for NK cell immunotherapy given that the *in vitro* effect of IL-2 on NK cells functions was not reflected *in vivo*.

7.6 Cytokine combination

The purpose of using combinations of cytokines in the present study was to assess whether certain cytokines have a synergistic effect on each other, as was indicated by several studies (de Rham et al., 2007, French et al., 2006a, Son et al., 2001, Strengell et al., 2003). The combinations that I studied were based on my initial findings regarding the phenotypes of cytokine-activated NK cells (Chapter 3). IL-15 was the most potent cytokine for inducing the expression of activating receptors on both PB and CB NK cells. However, IL-15 did not promote the expression of L-selectin on CB NK cells, a

receptor that is key in NK cell trafficking to LN. On the other hand, IL-12 or IL-18 were shown to upregulate the expression of L-selectin. In addition, IL-18 is known to upregulate CCR7 expression. Since IL-12 has been shown to be toxic with documented clinical adverse effects, I focused on the combinations of IL-15+IL-2 and IL-15+IL-18.

IL-15 is critical for NK cell development, homeostasis and activation. In this context, IL-15 may enhance the cytotoxicity of CB NK cells and promote their differentiation from CD34⁺ HSCs in a transplantation setting. The feasibility of using the combination of IL-15 with IL-2 to activate CB NK cells was shown here. First, IL-15+IL-2 induced the highest killing capacity by CB NK cells. Moreover, cytotoxicity of IL-15+IL-2 stimulated CB NK cells exceeds PB NK cells at the different time points examined here. Second, IL-15+IL-2 promoted significant proliferative capacity of CB NK cells. Third, this combination was shown to induce chemotaxis of CB NK cells. Importantly, both IL-2 and IL-15 are FDA approved and have already been used in clinical trials, thereby making this combination attractive for further investigations.

Similarly, the combination of IL-15+IL-18 was shown here to induce the production of inflammatory cytokines by CB NK cells, but also to enhance cytotoxicity upon prolonged stimulation. Nomura *et al.* showed previously that CB NK cells demonstrate selective responses to IL-18 stimulation (Nomura et al., 2001). I showed here that CB NK cells express less IL-18R than PB NK cells, which possibly explains their poor killing capacity when stimulated with IL-18 alone. In contrast, the use of IL-15+IL-18 may deliver a synergistic effect by: (i) upregulating IL-18R expression, (ii) promoting IL-15-mediated NK cell survival and (iii) enhancing both cytotoxicity and cytokine production by NK cells. IL-18 has already been used in clinical trials and was shown to be less toxic than other cytokines as no maximum tolerated dose has been detected yet (Robertson et al., 2006, Tarhini et al., 2009). Although IL-18 alone induces limited anti-tumour activity, its function can be exploited if used in combination with other cytokines. In this context, it has been shown recently that IL-18 in combination with IFN- α , IL-2, IL-12 or IL-15 promotes the production of CCL19 within tumour-associated lymphoid tissues and this provides a rationale for the use of IL-18 as a co-stimulating agent in an immunotherapeutic context (Wong et al., 2013).

In this study, I tested NK cell cytotoxicity *in vitro* using the K562 cell line. As the

cytotoxicity of cytokine-activated NK cells against primary AML cells would be an interesting approach to determine which conditions promote maximal activity of PB and CB NK cells, as NK cell activity correlates with the clearance of AML blasts after HSCT (Lion et al., 2012). In addition, the functionality of cytokine-activated NK cells *in vivo* remains to be investigated using an animal model. In this context, K562 or primary AML cells could be injected into irradiated mice along with cytokine-activated NK cells to further investigate their cytotoxicity. The ideal mouse model to study NK cell cytotoxicity *in vivo* is using NOD-*scid- γ ^{-/-}* mice as this strain lacks T cells, B cells and functional NK cells, exhibits enhanced engraftment of human lymphocytes and supports the growth of many transplantable human tumour cells (Shultz et al., 2007). NOD-*scid- γ ^{-/-}* mice can be irradiated and intravenously injected with GFP-labelled tumour cells as has been shown by Luevano and colleagues (Luevano et al., 2014). Twenty four hours post-tumour injection, cytokine-activated NK cells can be injected into mice and their localisation into various tissues including liver, spleen, lungs and BM assessed 2 days later. Mice injected only with tumour cells can be used as a control in this experimental setting. This model can be helpful to assess cytotoxicity of NK cells *in vivo* as well the accumulation of NK cells in certain tissues after adoptive transfer.

7.7 Do NK cell traffic to tumour sites after adoptive transfer?

Successful approaches for adoptive transferring of cytokine-activated NK cells should offer effective recruitment of NK cells to inflammatory or tumour sites. In this context, the expression of CCR7 and/or CXCR3 mediates NK cell trafficking towards LN and tumour sites and hence the correct localisation to kill cancerous cells. Herein, I demonstrated upregulation of chemokine receptors involved in NK cell trafficking to LN and inflamed tissues as well as migration of PB and CB NK cells after IL-15+IL-2 or IL-15+IL-18 activation *in vitro*. CXCR3 expression was upregulated on PB and CB NK cells after cytokine stimulation. *In vivo*, CXCR3⁺ NK cells are only able to infiltrate tumour tissues when CXCR3 ligand (CXCL10) is expressed. The upregulation of CXCL10 was shown to be IFN- γ -dependent (Wendel et al., 2008). In this context, I showed here that NK cells secreted high concentrations of IFN- γ after stimulation with cytokines, in particular IL-15+IL-18. Hence, it is possible that IFN- γ production by IL-

15+IL-18 activated NK cells could contribute to the upregulation of CXCL10 and enhanced NK cell trafficking to tumour sites or LN.

Currently, the trafficking repertoire of adoptively transferred NK cells is not fully understood. A recent study has shown that adoptively transferred murine NK cells are efficiently recruited and accumulated within tumour sites (Gill et al., 2012). However, this study demonstrated that NK cells lost their capacity to attack tumour cells at day 5 after transfer, and that they downregulated the expression of activating receptors, suggesting exhaustion of the transferred NK cells. Whilst it would be interesting to assess migration of cytokine-activated NK cells *in vivo*, this may be difficult as NK cell trafficking mechanisms vary between humans and mice (Martín-Fontecha et al., 2004).

7.8 Memory-like NK cells

It has been shown recently that cytokine stimulation induces memory-like properties in PB NK cells (Cooper et al., 2009, Ni et al., 2012, Romee et al., 2012). Hence, I tested the feasibility of generating memory-like NK cells from CB. My initial findings showed that CB NK cells respond to some cytokines even better than PB NK cells. I was able to generate memory-like NK cells from PB and CB NK cells with sustained ability to produce IFN- γ after 21 days of culture after cytokine stimulation. This work is significant in the context of NK cell-based immunotherapy, as the ability to maintain NK cells in long-term culture offers a flexible source for multiple NK cell infusions from the same donor. Furthermore, the fact that these cells are a rich source of IFN- γ could be utilised to treat viral infections post-transplantation. Indeed, Foley and colleagues showed this using different allogeneic transplantation cohorts: T cell-deplete, T cell-replete from unrelated BM or PB donors and CBT. They showed that reconstituted NK cells have impaired IFN- γ production particularly in T cell-depleted and CB cohorts suggesting that T cells play an important role in NK cell education (Foley et al., 2011). Hence, cytokine-induced memory-like NK cells may be infused in HSCT recipients to provide protection from post-transplantation infections.

The cytotoxic capacity of cytokine-induced memory-like NK cells was not assessed here as it was shown firstly by Cooper and colleagues that the cytotoxic capacity of

memory-like NK cells was not enhanced in this protocol (Cooper et al., 2009). However, Ni *et al.* showed that the infusion of IL-12+IL-15+IL-18 pre-activated NK cells, but not naïve or IL-2 nor IL-15 stimulated NK cells, resulted in substantial reduction of tumour growth (Ni et al., 2012). Moreover, cytokine pre-activated memory-like NK cells have been shown to persist for up to 3 months following adoptive transfer. Interestingly, cytokine pre-activation results upregulates the expression of the IL-2R α by memory-like NK cells. As a result, the interaction between T helper cells and cytokine pre-activated NK cells within LN leads to robust expansion of memory-like NK cells, promoting their anti-tumour activity and priming of T cell specific responses (Ni et al., 2012, Leong et al., 2014). Given that cytokine pre-activated NK cells undergo modification of cytokine receptor expression, further analysis of cytokine receptors, in particular IL-2R α and phosphorylation of STAT5 components, should be carried out, especially because I showed in this study that CB NK cells express lower levels of IL-2R α . Also, further analysis of the chemokine receptor repertoire remains to be done as cytokine-induced memory-like NK cells were shown by Ni and colleagues to accumulate at tumour sites and produce IFN- γ . In addition, *in vitro* and *in vivo* cytotoxic function of cytokine-induced memory-like NK cells against K562 or primary AML cells remains to be tested. Romee and colleagues have injected cytokine-induced memory-like NK cells into NOD-*scid*- γ c^{-/-} mice to study their expansion and accumulation within specific tissues (Leong et al., 2013). The expansion of transferred NK cells in this model was supported by low dose administration of IL-2. Hence, similar models could be utilised in future experiments to investigate expansion and cytotoxicity of cytokine-induced memory-like NK cells *in vivo* and importantly to provide comparative analysis of memory-like NK cells generated from the PB and CB sources.

So far, the best-characterised model for memory-like NK cell properties has been for MCMV infection. Memory-like NK cells showed specific recall responses to m175 MCMV derived proteins that are recognised by Ly49 on mouse NK cells (Sun et al., 2009), whereas NKG2C⁺ NK cells expand in humans as a result of viral infections to mediate anti-viral protection (Gumá et al., 2006, Lopez-Vergès et al., 2011). In contrast to viral-induced memory-like NK cells, cytokine-induced memory-like NK cells do not confer specificity towards a particular antigen. Instead, cytokine-induced memory-like NK cells are a rich source of IFN- γ and are readily responsive to restimulation. Thus,

their *in vivo* function can be studied in an HSCT context to investigate whether cytokine-induced memory NK cells mediate anti-viral protection, in particular to CMV infection, post-transplantation. Humanised mouse models have been developed by transplantation of HSCs into immunodeficient mice providing a tool to study human lymphocyte differentiation, reconstitution and also potential to investigate tumour growth and pathogenesis of infectious diseases (Legrand et al., 2009). Recently, the first humanised model of HCMV has been developed by transplantation of HSCs from CMV seropositive donors into NOD-*scid- γ c*^{-/-} mice, in which mice demonstrated evidence of HCMV in the liver, spleen and BM (Hakki et al., 2014). Such a model could be utilised by subsequent infusion of cytokine-induced memory-like NK cells to assess whether these cells confer IFN- γ -mediated anti-viral protection. Currently, the infusion of cytokine-induced memory-like NK cells from a haploidentical donor is being tested in a phase I clinical trial to treat refractory AML by Romee and colleagues (clinicaltrials.gov, trial number; NCT01898793). It is possible that the cytokine-induced memory-like NK cells generated here from CB may offer a better option given that CB is a more readily available cell source.

7.9 Cytokine dependence

The expansion and cytotoxic function of *ex vivo* cytokine-activated NK cells following adoptive transfer needs to be supported by exogenous cytokine administration leading to the concept of cytokine addiction. Basically, NK cells exposed to high doses of cytokines *ex vivo* renders them susceptible to apoptosis when the cytokine doses are reduced *in vivo*. Berg and colleagues showed that IL-2 removal from the culture medium results in a sharp decline in the percentage of NK cells expressing NKG2D and TRAIL, while the expression of these markers was restored after subsequent addition of IL-2 to the culture in a dose-dependent manner (Berg et al., 2009). Miller and colleagues have reported similar findings, in which expansion of IL-2 or IL-15 activated NK cells was reduced by 90 % 1 week after discontinuing cytokine administration. In this context, it was suggested by the same group that IL-15 might be better than IL-2 in promoting expansion of adoptively transferred NK cells (Miller, 2013). In line with these findings, my results showed that *in vitro* proliferation of NK cells activated with IL-15 or its combination with IL-2 is superior to those expanded with IL-2 alone, especially for CB NK cells. Hence, similar mechanisms may take place

in vivo and survival of adoptively transferred NK cells could be supported with low doses of IL-15. IL-15 could be recycled by DCs and represented via IL-15R α to NK cells mediating their survival even after IL-15 withdrawal (Dubois et al., 2002). In addition, NK cells may utilise the expression of IL-15R α in immunotherapeutic settings without the involvement of DCs by enhancing IL-15 presentation in an autocrine manner. My results showed that resting CB NK cells express significantly higher IL-15R α than PB NK cells. Thus, IL-15R α could be blocked using monoclonal antibodies to assess the role of this receptor in NK cell responsiveness to exogenous IL-15 by PB and CB NK cells in future comparative experiments, in particular NK cell expansion in response to IL-15 or IL-15 combinations.

7.10 Therapeutic implications of CB NK cells

NK cells are a promising therapeutic cellular source to treat cancers or to improve the outcomes after HSCT. Based on Ruggeri hypothesis, KIR ligand incompatibility between donor and recipient induces NK cell alloreactivity, whereby NK cells become more potent to attack leukemic cells (Ruggeri et al., 2005). Hence, allogeneic NK cells offer a promising source for cancer immunotherapy mediating GvL. CB is a potential source of NK cells as it has advantages over PB including immediate availability; more tolerance of HLA disparity, reduced intensity and severity of GvHD and possible GvL effect (Verneris et al., 2009). A study to evaluate the outcomes of KIR ligand incompatibility in CBT context showed that relapse rate was lower among KIR mismatched CB recipients (Miller et al., 2006). Béziat et al. have shown similar findings as relapse rates were lower after CBT and have shown that reconstituted CB NK cells have functional maturity, and importantly suggested that CB NK cells may mediate GvL post CBT (Béziat et al., 2009). However, the beneficial effect of KIR ligand incompatibility remains controversial although a Eurocord study showed favorable results in terms of leukaemia-free survival and relapse rates in CBT settings (Willemze et al., 2010). Given this, CB NK cells are possibly as potent as PB NK cells for allogeneic NK cell adoptive transfer. However, a challenge remains to consider, the expansion of CB NK cells with an activated phenotype for adoptive transfer to ensure efficacy of CB NK cells against cancerous cells.

Several trials are underway to exploit the function of CB NK cells, primarily for

haematological malignancy treatment. CB NK cells are infused along with CBT to enhance GvL effect and reduce GvHD improving the outcomes of transplantation in leukaemia, lymphoma and multiple myeloma patients (clinicaltrial.gov, trial number; NCT01619761). In addition, CB NK cells can be expanded *ex vivo* with IL-2 and infused into multiple myeloma patients post chemotherapy and post-HSCT to kill residual myeloma cells (clinicaltrial.gov, trial number; NCT01729091). Another trial is aiming to treat refractory hematologic cancers by performing TBI followed by CBT and infusion of CB NK cells together with subcutaneous injections or systemic administration of IL-2 to support their expansion *in vivo* (clinicaltrial.gov, trial numbers; NCT00354172 and NCT00871689). Finally, a phase I clinical trial aims to activate mixed populations of CB NK cells and T cells with variety of cytokines to prevent tumour relapse after radical resection of the tumour in the context of hepatocellular carcinoma, renal cell carcinoma, lung cancer (clinicaltrial.gov, trial number; NCT01914263).

Altogether, an NK cell therapy using CB as a cell source has potential in particular post-HSCT to prevent GvHD and improve GvL. Although the majority of trials and studies focuses on the use of CB NK cells to improve HSCT outcomes in haematological malignancies context, future studies may involve using of CB NK cells for the treatment of solid tumours. So far, anti-cancer effect of NK cells is mainly seen against AML blasts indicating the need to optimise NK cell-based therapy, via expansion or conditioning regimes to allow broader use of NK cells. Future implication of CB NK cells would certainly utilise cytokines to induce effector functions of CB NK cells as resting CB NK cells are poorly cytotoxic.

7.11 Translation to the clinic

In this study, I have shown that short periods of NK cell stimulation with cytokines promotes immediate cytotoxic functions by PB and CB NK cells and that the generation of memory-like NK cells provides a cellular source that can be maintained for prolonged periods *in vitro*. Theoretically, the application of either source would be dependent on the underlying condition as well as the accessibility of NK cell sources. NK cells upregulate L-selectin and CCR7 expression within short periods of stimulation with cytokines and might have the potency to home to LN. Indeed, it is known that prolonged stimulation of NK cells results in downregulation of L-selectin expression by

NK cells hence limiting their capability to home to LN (Romee et al., 2013). Also, it has been shown that CCR7 expression declines after 72 hours of uptake by trogocytosis (Somanchi et al., 2012), whereas the option of maintaining memory-like NK cells in prolonged cultures could be appealing for multiple infusions of NK cells into the same patient.

As NK cells are the first lymphocyte population to reconstitute after HSCT it is believed that they play a key role in mediating GvL and reducing the incidence of GvHD. Many on-going trials are underway to treat AML and other haematological malignancies by promoting the GvL effect via NK cell infusion after HSCT (clinicaltrial.gov, trial numbers; NCT01220544, NCT00625729, NCT01853358). The aim of these trials is to potentiate donor NK cell-mediated killing of cancer cells by infusion of *ex vivo* expanded NK cells following HSCT. Such an approach delivers multiple functions of NK cells: (i) *ex vivo* cytokine-activated NK cells are more potent at targeting residual tumour cells after chemotherapy, (ii) infused NK cells reduce the incidence of GvHD by inhibition of donor alloreactive T cells (Olson et al., 2010), and (iii) reconstituted NK cells have the potency to become alloreactive according to the KIR-KIR ligand incompatibility hypothesis (Ruggeri et al., 2005).

Although IL-2 has been used widely to activate NK cells in clinical trials, IL-15 has proven to be indispensable for NK cell functions *in vivo*. Currently, Miller and colleagues are assessing the systemic administration of IL-15 in conjunction with NK cell infusion from a haploidentical donor to treat refractory AML (clinicaltrial.gov, NCT01385423). As I showed in this study, stimulation with IL-15+IL-2 or IL-15+IL-18, promoted substantial cytotoxic functions of CB NK cells. Hence, my findings suggest that CB NK cells should be stimulated with IL-15+IL-2 or IL-15+IL-18 combinations rather than IL-2 alone in order to be used as a cellular immunotherapeutic source to achieve optimal anti-tumour activity.

In the present study, cells were stimulated and expanded in 96-well culture plates. Thus, protocols to optimise the product to GMP standards are still needed in order to make cytokine-activated NK cells suitable for clinical use. Recently, Koehl and colleagues described a protocol for clinical scale NK cell enrichment using GMP procedures (Koehl et al., 2013). This approach produces highly purified NK cell products whilst

maintaining cytotoxic function and importantly with minimal T cell contamination. This is performed using a fully closed system (bioreactors) and CliniMACS reagents designed to deplete $CD3^+$ cells followed by enrichment of $CD56^+$ cells. The purified NK cells can then be expanded using cytokines (Fig 7.1). Hence, this protocol seems to be applicable for CB units given that CB is readily available and is a rich source of NK cells that are responsive to cytokine stimulation. The cytotoxicity of isolated CB NK cells could be further enhanced using a combination of IL-15+IL-2 or IL-15+IL-18 instead of using IL-2 alone. Thereafter, medium could be changed every three days and then the product infused into a recipient at different intervals or cryopreserved for future use. Expanded NK cells can be infused into HSCT recipients to kill residual tumour cells after transplantation or into solid tumours to promote an NK cell anti-tumour effect.

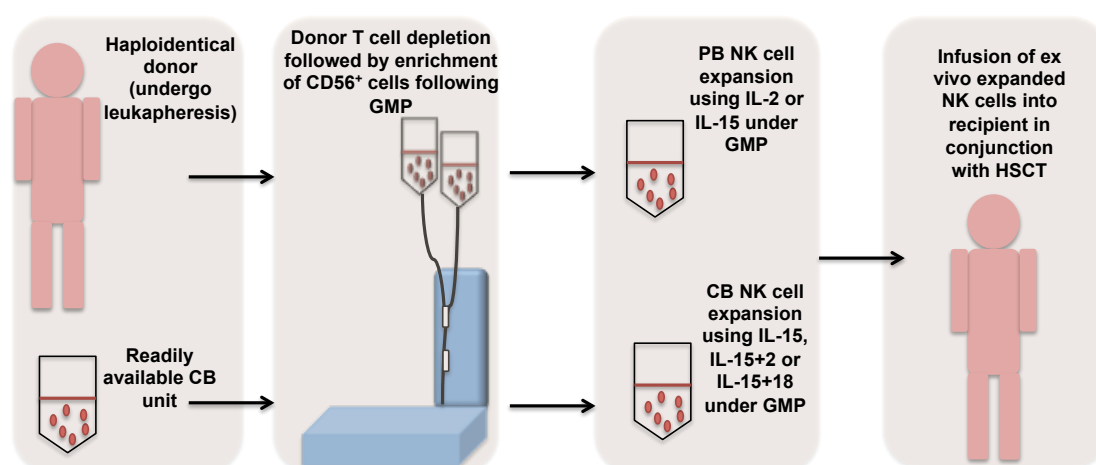


Figure 7.1 A model of adoptive transfer of *ex vivo* cytokine-activated NK cells into HSC recipients and solid tumour patients. NK cells can be enriched from a haploidentical donor or CB units and expanded with cytokines following GMP procedures. Expanded NK cells can be infused to treat different type of malignancies or stored for future use. Adapted from “Clinical grade purification and expansion of NK cell products for an optimized manufacturing protocol” by Koehl and colleagues (Koehl et al., 2013).

In conclusion, differential activation of PB and CB NK cells using different cytokine combinations have been identified in the current study. PB NK cells showed optimum activation following stimulation with IL-2 whereas CB NK cells showed the least responsiveness to IL-2 stimulation among other cytokine conditionings. Less responsiveness of CB NK cells to IL-2 stimulation was associated with reduced expression of IL-2 receptors and reduced phosphorylation of STAT5 pathway

components. On the other hand, CB NK cells responded better to IL-15+IL-2 or IL-15+IL-18 stimulation than IL-2 alone. The combination of IL-15+IL-2 promoted maximal cytotoxicity of CB NK cells whereas the combination of IL-15+IL-18 induced high levels of IFN- γ and TNF- α secretion. Furthermore, the use of either combination enhanced PB and CB NK cells migration towards LN chemokines *in vitro*. In addition I was able to generate memory-like NK cells from PB and CB NK cells using cytokines with sustained IFN- γ production for 21 days of culture, offering a future source of NK cells for immunotherapy. These findings provide the basis for the activation of NK cells derived from different sources using cytokines and could be considered in the future for NK cell-based immunotherapy. The data presented in this study stipulates the extension of this knowledge into animal models to gain a more complete understanding of cytokine-activated NK cell functionality *in vivo*. Overall, cytokine activation induces fully functional CB NK cells. Together with other advantages of CB including off-the-shelf availability and less stringent HLA-matching, CB NK cells are potentially superior to PB NK cells as a cellular therapeutic source.

Chapter 8: Appendices

8.1 Presentations

- UK NK meeting, London 2012
- 4th King Abdullah International Medical Research Centre Annual Forum, Riyadh 2013

8.2 Poster presentations

- British Society for Immunology Congress, Liverpool 2010
- NK2012, Heidelberg 2012
- NK2013, Heidelberg 2013

8.4 Publications

- Luevano M, Daryouzeh M, Alnabhan R, Querol S, Khakoo S, Madrigal A, et al. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Human Immunology*. 2012;73(3):248-57.
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